Inhibition of Castration Resistant Prostate Cancer by Sintokamide A: an Antagonist of the Amino-Terminus of the Androgen Receptor

by

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Abstract

The majority of lethal castration resistant prostate cancer (CRPC) is considered to involve a transcriptionally active androgen receptor (AR). All current therapies target AR ligand-binding domain (LBD) to inhibit receptor activity. Unfortunately these therapies fail perhaps by mechanisms involving expression of constitutively active AR splice variants that lack LBD and/or *de novo* androgen synthesis. Recent studies have provided evidence that AR N-terminus domain (NTD) is druggable with the development of EPI-001, an antagonist that has specificity and efficacy on CRPC xenografts. Here we reveal an unrelated class of compounds, SINT1 as a potent therapeutic agent for CRPC that targets NTD.

To examine the effects of SINT1 on AR transcriptional activity, endogenous gene expression and reporter constructs regulated by AR were examined. The ability of SINT1 to directly inhibit transactivation of the NTD was tested using a chimera of NTD fused to the Gal4DBD. Reporter assays for glucocorticoid (GR) and progesterone receptor (PR) were measured to ensure specificity. Combination study of SINT1 and EPI-002 was performed by measuring AR transcriptional activity. BrdU incorporation was analyzed to indicate changes in proliferation. Effect of SINT1 on AR N/C interaction was assessed using a 2-hybrid assay. Animals bearing LNCaP xenografts were castrated and randomized into two groups. One week after castration, the animals were treated every 3 days with an intratumoral dose of SINT1 (30 mg/kg) or vehicle.

SINT1: blocked AR activity as measured by reduction in PSA mRNA and reporter activity induced by androgen; reduced transactivation of AR NTD to baseline levels; had no effects on

transcriptional activities of related steroid receptors; was effective in blocking androgen-induced proliferation in LNCaP cells but not PC3 cells; caused an increase in caspase-3/7 activity; showed an additive inhibitory effect to EPI-002; reduced AR N/C interaction; regressed some CRPC xenografts with no change in animal body weight compared to DMSO-treated tumors.

Together these data support that SINT1 is a specific inhibitor of the AR NTD without effects on highly related steroid hormone receptors and no apparent toxicity in animals. SINT1 is an antagonist to AR NTD that causes regression of CRPC xenografts.

Preface

Sintokamides were co-discovered by Dr. Raymond Andersen and Dr. Marianne Sadar. The chemical structures of sintokamides were identified and named in the laboratory of Dr. Raymond Andersen at UBC Department of Chemistry. All chemical characterization of sintokamides were done by Dr. Andersen's team as well. All biological characterizations of sintokamides and their implication in prostate cancer research were done by Dr. Marianne Sadar's group. Details of discovery and characterization of sintokamides has been published in: Sadar, M.D., et al., *Sintokamides A to E, chlorinated peptides from the sponge Dysidea sp. that inhibit transactivation of the N-terminus of the androgen receptor in prostate cancer cells.* Org Lett, 2008. **10**(21): p. 4947-50.

In this thesis:

Dr. Marianne Sadar designed and supervised all experiments and the overall project. Dr. Sadar and the members of my supervisory and examination committees (thanked in the acknowledgement section) provided critical feedback for the content and editing of this thesis.

Chapter 1: This chapter was written entirely by Iran Tavakoli by a comprehensive search of the literature on the related subjects. Sections of figure 1, figure 3A, figure 4, and their captions are republished from the articles below. The permission of the senior author and agreement of the publishers were obtained for using a figure from these articles:

Figure 1: Waltering, K.K., A. Urbanucci, and T. Visakorpi, *Androgen receptor (AR) aberrations in castration-resistant prostate cancer.* Mol Cell Endocrinol, 2012.

Figure 3A: Kumar, R. and I.J. McEwan, *Allosteric Modulators of Steroid Hormone Receptors: Structural Dynamics and Gene Regulation.* Endocrine Reviews, 2012. **33**(2): p. 271-299.

Figure 4: Sadar, M.D., et al., *Sintokamides A to E, chlorinated peptides from the sponge Dysidea sp. that inhibit transactivation of the N-terminus of the androgen receptor in prostate cancer cells.* Org Lett, 2008. **10**(21): p. 4947-50.

Chapter 2: I (Iran Tavakoli) was the main contributor to this work. I performed most of the experiments, analyzed the results, prepared the graphs, and wrote the entire chapter. I performed AR N/C interaction, EPI-002 and SINT1 combination study, AR nuclear translocation, caspase-3/7 assay, and AR ligand binding assay. Steroid receptor specificity assay was performed by Teresa Tam. The qRT-PCR assay was done by Theresa Yong. Kevin (Yu Chi) Yang assisted with the set up and performance of the AR ligand binding assay. AR NTD transactivation and proliferation studies were performed by Nasrin R. Mawji and Teresa Tam, and the results of these experiments have been published previously in the paper mentioned above. Studies confirming these results were incorporated into the figure 5 in this thesis to help with the flow of the thesis according to the UBC thesis requirements.

Pharmacokinetics study and resulting data analysis were done at *NAEJA Pharmaceuticals Inc.* Dr. Marianne Sadar designed the LNCaP tumor xenografts study presented in figure 10, and Jean Wang performed the animal work. All data were analyzed and figures prepared by Iran Tavakoli. Immunohistochemistry was done at *Wax-it histology services Inc*. and results were analyzed by Iran Tavakoli.

Chapter 3: This chapter was written entirely by Iran Tavakoli. All ideas and discussions are based on the search of the literature and interpretation of the findings presented in this thesis.

The use of animals for the experiments was approved under application number A09-0474 by the University of British Columbia Animal Care and Use Committee. The use of

biohazard material was approved under protocol number B07-0170 by the University of British Columbia Biohazards Committee.

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List of Abbreviations

ADT	androgen deprivation therapy
AF	activation function
AIS	androgen insensitivity syndrome
AR	androgen receptor
ARE	androgen response element
BIC	bicalutamide
CBP	CREB binding protein
ChIP	chromatin immunoprecipitation
CRPC	castration resistant prostate cancer
CTRL	control
DBD	DNA binding domain
DHT	dihydrotestosterone
DMEM	Dulbecco's modified eagle medium
EGF	epidermal growth factor
FBS	fetal bovine serum
FSK	forskolin
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
GF	growth factor
GM-CSF	granulocyte-macrophage colony stimulating factor
GnRH	gonadotropin releasing hormone
GR	glucocorticoid receptor
ID	intrinsically disordered
IDP	intrinsically disordered protein
IGF	insulin growth factor
IHC	immunohistochemistry
IL-6	interleukin-6
KLK3	kallikrein 3
LBD	ligand binding domain
LH	luteinizing hormone

LNCaP	lymph node carcinoma of the prostate cell line
MAPK	mitogen activated protein kinase
MEM	minimum essential medium
NCoR	nuclear receptor co-repressor
NLS	nuclear localization signal
NMR	nuclear magnetic resonance
NTD	N-terminal domain
PARP	poly (ADP-ribose) polymerase
PI3K	phosphatidylinositol 3-kinases
PKA	protein kinase A
PR	progesterone receptor
PSA	prostate specific antigen
PTEN	phosphatase and tensin homolog
PTM	post translational modification
qRT-PCR	quantitative real-time polymerase chain reaction
RPMI	Roswell park memorial institute
SAR	structure activity relationship
SCID	severe combined immunodeficiency
SHR	steroid hormone receptor
SINT1	sintokamide A
SMRT	silencing mediator for retinoid or thyroid-hormone receptors
SRC	steroid receptor co-activator
TAU	transactivation unit
TF	transcription factor
TSS	transcription start site
UTR	un-translated region
YFP	yellow fluorescent protein

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Chapter 1 – Introduction

1.1 Prostate cancer

1.1.1 Epidemiology

Prostate cancer is the most frequently diagnosed cancer among Canadian men (excluding non-melanoma skin cancer). An estimated 25,500 men were diagnosed and 4,100 died of this disease in 2011. On average, one in seven men will develop prostate cancer during his lifetime, and one in 28 will die of it. An overall rising trend in the incidence of prostate cancer has been observed since 1980, but death rates started to decline in the mid 1990s, mainly due to the advances in early diagnosis of the disease [1]. Age, family history, and African ethnicity are established risk factors for prostate cancer [2]. Other risk factors of prostate cancer include smoking and alcohol consumption. Recent studies have established a beneficial role for physical activity and diet for incidence of cancer and quality of life after diagnosis [3-5].

1.1.2 Diagnosis

The screening method used for early diagnosis of prostate cancer is digital rectal examination and a blood test for levels of prostate-specific antigen (PSA) [6, 7]. PSA is a serine protease secreted by prostate epithelial cells as part of the seminal fluid [8]. This biomarker has been used since the early 1990s, and its levels in the serum have been shown to correlate with volume of tumor and clinical stage of the disease [9]. The PSA gene has androgen response elements (AREs) in its promoter and enhancer regions, hence it is known as an androgen-regulated gene [10]. As a result, serum PSA levels decline rapidly in response to androgen ablation therapy. However, the subsequent rise in levels of PSA is indicative of progression to

advanced prostate cancer [11]. Although PSA levels are also influenced by other prostate disease conditions, such as benign prostate hyperplasia, prostatitis, age and race, PSA is still used as a sensitive marker for screening, diagnosis, and monitoring the treatment of advanced prostate cancer [12]. Definitive diagnosis of prostate cancer is established based on biopsy results through assessment of the histopathology of tissue. The biopsy of the prostate is performed under transrectal ultrasound guidance and provides key information about tumor grade, volume and staging [13]. The appropriate treatment for the patient is determined based on a comprehensive consideration of the tumor grade and stage, age and health status of the patient [14].

1.1.3 Hormonal progression of prostate cancer

The majority of localized early stage prostate tumors are treated successfully by radical prostatectomy [15, 16], external-beam radiotherapy [17, 18], or more recently with brachytherapy [19] which achieves more specific targeting and less side effects. However, in about 30% of patients, their tumors progress to invasive and disseminated disease. For those patients with metastasis or advanced prostate cancer, androgen deprivation therapy (ADT) through either chemical or surgical castration is the general treatment. In 1966, the Nobel Prize in medicine was awarded to Canadian Charles B. Huggins for his work in demonstrating the beneficial effects of ADT for patients with metastatic prostate cancer. His work laid the foundation for the current standard approach of androgen blockade through surgical and/or medical castration [20]. ADT is based on the recognition of the role of androgens in prostate carcinogenesis [21]. Androgens are small four ringed planar molecules made from cholesterol precursor that play a critical role in the development of the prostate gland and male phenotype in embryogenesis [22]. Testosterone and its more potent metabolite dihydrotestosterone (DHT) both cause a cellular response to hormone in the prostate [23]. Androgens mediate their effect by

binding the androgen receptor (AR) in prostate cells. In the absence of androgens, AR is cytoplasmic in a complex with chaperone and immunophilin proteins [24, 25]. Upon ligand binding, AR undergoes conformational changes and translocates to the nucleus. AR binds the AREs and interacts with co-regulatory molecules including general transcription machinery and histone modifying enzymes, and mediates expression of androgen-regulated genes (discussed in more detail later) [26]. This AR signaling causes the cellular response to the hormone. Hence selection of targets along the endocrine and cellular axis of androgen functions is a critical part of ADT. This includes the hypothalamic–pituitary–testicular–adrenal axis for androgen synthesis and secretion as well as the AR signaling pathway within the prostate cancer cell including the receptor-protein and receptor-DNA interactions. Therapies disrupting this axis that have been approved for ADT include: 1) luteinising hormone-releasing hormone (LHRH) agonist/antagonist such as goserelin acetate and degarelix acetate; 2) nonsteroidal AR antagonists (known as antiandrogens) such as hydroxy flutamide, bicalutamide and nilutamide; 3) steroidal antiandrogens such as cyproterone acetate; 4) adrenal androgen inhibitors such as ketoconazole; and 5) inhibitors of 5α-reductase, an enzyme that converts testosterone to DHT, such as finasteride. Abiraterone acetate, a novel inhibitor of CYP17 enzyme, has recently been approved for post chemotherapy treatment. Clinical trials are ongoing to investigate the effect of this compound for ADT (discussed in more detail later) [27]. Initial responses to ADT are generally successful but all therapies will fail and lethal castration-resistant prostate cancer (CRPC) will emerge and this stage of the disease is incurable [28].

CRPC or advanced prostate cancer was previously known as “androgen independent” disease. However, new evidence has revolutionized the prostate cancer research field by redefining this term. Studies have shown that gonadal androgens only account for up to 80% of

circulating androgens. Other sources of androgen synthesis are adrenal glands, and presence of adrenal androgens in the tumors has been confirmed in clinical samples from CRPC patients [29]. Hence castration does not block the androgen synthesis from the adrenal glands [30]. New studies also confirm that tumor cells are capable of synthesizing estrogen and androgen from other precursors [31]. This evidence suggests that androgens and AR signaling continue to be an integral part of disease progression in advanced prostate cancer. Hence the term “androgen independent” was changed to “castration-resistant” prostate cancer in view of the dependency of the disease on androgens [30].

However upregulation of androgen synthesis is only a part of the spectrum of CRPC mechanisms. Evidence suggests that AR dysregulation also plays a key role in CRPC progression. AR dysregulation is thought to occur through mechanisms including: 1) amplification or over expression of AR; 2) gain-of-function somatic mutation of AR; 3) aberrant AR post-translational modification (frequently driven by growth factor (GF) or cytokine signaling); 4) alternative splicing events that result in constitutively active receptors; and 5) co-regulatory proteins dysregulation [32, 33].

Based on this new evidence, deciphering the role of AR signaling and its regulation as well as identifying the key enzymes in intra-tumoral androgen maintenance has become the focus of drug development for CRPC. Discovering two groups of compounds has received particular attention: first, more potent inhibitors of AR transcriptional activity and, second, inhibitors that target the synthesis of androgenic ligands [28]. Examples of novel drugs for CRPC treatment are discussed later.

1.2 Androgen receptor

1.2.1 The structure of the AR

AR is a member of the nuclear receptor super family which are intracellular ligand-activated transcription factors [34]. The AR gene is located on chromosome Xq11-12 and spans ~180 kb of DNA containing 8 canonical exons, and encodes a 919 amino acids protein [23, 35]. Novel exons that span introns flanking the canonical exons that encode the AR splice variants, as well as a novel exon that maps to the 3′ untranslated region (3′ UTR) have recently been reported [36-38]. However, in this study, the 8 canonical exons are mainly discussed. Similar to other steroid hormone receptors, AR contains functional domains, including: the N-terminal domain (NTD) that modulates transcription activation [35]; a DNA-binding domain (DBD) that binds to ARE in target gene sequence; a hinge region and a C-terminal ligand-binding domain (LBD) (

Figure 1) [34]. A nuclear localization signal (NLS) spans the region between the DBD and the hinge region. AR has 2 activation function regions that play critical roles in its transcriptional activities. The activation function 1 (AF1) is located at the NTD and plays the primary role in transactivation of target genes [39-41]. AF2 is located in the AR LBD and is critical for protein-protein interactions [42]. Deletion studies have shown that this activation domain shows limited transactivation function in the absence of NTD [39, 40, 43]. Whether AF1 and AF2 surfaces have selective roles and mediate different gene expressions is an interesting question that is currently under investigation [44].

The crystal structure of the AR DBD [45] and LBD [23, 46-48] have been resolved. AR LBD shares similar structure with other steroid receptors [34], as it contains approximately 12 α-helices (H) and two β-turns that are arranged in three layers to form an anti-parallel ‘α-helical

sandwich' [49-51]. Upon agonist binding, H12 is repositioned and serves as the 'lid' of the ligand binding pocket to stabilize the ligand [52]. This conformational change creates a hydrophobic pocket on the LBD which makes up the AF2. This surface is important for protein-protein interactions that regulate receptor activity [49-51, 53, 54]. The surface of AF2 is also important for interaction with AR NTD [55, 56]. AR AF2 functions in a ligand-dependent manner and has sequence homology and conserved sequences to other steroid hormone receptors [34]. One unique feature of AR AF2 is that N/C interaction is preferred to interaction with co-regulatory proteins due to preference for bulkier residues in the NTD [55, 56].

The availability of a number of AR LBD crystal structures allows rational 'structure-based' drug design, to facilitate the rapid discovery and development of AR ligands. Hence, LBD has been an efficient target site for inhibiting the AR function. Antiandrogens bicalutamide and MDV3100 target the AR LBD [57, 58]. A rationale for focusing on modulation of AR LBD for drug discovery has been that ligand-binding can not only regulate AF2 function directly, but also regulate AF1 action indirectly via N/C interaction [59-62]. However, as discussed later, AR can be activated through other signaling pathways induced by cytokines and growth factors [63].

AR DBD consists of two zinc finger structures that form two α-helices, one of which sits on the DNA and forms hydrogen bonds with the nucleotides in the binding site [45]. Evidence from work with other steroid receptors demonstrates that DNA can also act as a modulator for receptor activity [64]. Binding different DNA sequences changes the structure of DBD and can be a surface for recruiting different co-regulatory proteins [44, 64].

1.2.2 AR nuclear translocation and androgen regulated genes

Unbound AR is mainly located in the cytoplasm and associated with chaperone heat shock proteins, hsp 90, 70 and 56 and immunophillin proteins [65, 66]. AR LBD is known to be critical for interacting with heat shock proteins [65]. Natural ligands are lipid soluble androgens [67] that diffuse into the cell and bind the AR. Upon ligand binding, AR dissociates from heat shock proteins, undergoes conformational changes including dimerization, gets phosphorylated [63, 68], and translocates to the nucleus through interaction of the NLS with α-importin protein (Figure 2) [24, 25]. AR LBD has also been shown to be the surface for receptor dimerization [68]. Of note is the importance of AR nuclear translocation for receptor activity [24]. Nuclear AR is in the position to activate target genes [69, 70], hence a unique mechanism for AR inhibition is blocking this translocation or selecting targets that do not cause AR nuclear translocation in the absence of ligand. Antiandrogens such as bicalutamide cause AR nuclear translocation, however next generations antiandrogens such as MDV3100 result in less translocation [57]. Once nuclear, AR can regulate target gene transcription by binding to AREs [71]. These ARE sequences, which are partial, palindromic or direct repeat of 6 bp, can be located upstream or downstream of the transcription start site (TSS) of the target genes in the promoter or enhancer. AR also interacts and recruits other co-regulatory proteins including coactivators and corepressors [71, 72] for regulation of thousands of AR regulated target genes [71].

Recent ChIP-Chip or ChIP-seq studies in cell lines have shown that the receptors not only bind clearly recognizable AREs, but also bind other sites such as imperfect palindromic sequences and half sites [73-76]. These studies also confirm that the hormone receptor binding sites are located within 50 kb of TSS, and interestingly, composite sites that allow for binding of

multiple transcription factors are located in these regions [73-76]. Studies on how the interaction of multiple transcription factors allows for selective gene expression are ongoing.

Consistent with the function of AR, in drug development studies, the levels of common AR target gene transcripts including PSA and TMPRSS2 are assessed to show the inhibitory effect of drugs on endogenous gene expression. PSA, also known as kallikrein 3 (KLK3), is a 34 kD glycoprotein [8]. Production of this serine protease enzyme is exclusive to the prostatic epithelial cells. PSA expression is a measure for prostate specific AR transcriptional activity [77]. TMPRSS2, transmembrane protease serine 2, is a transmembrane protein [78] also known to be regulated by AR in response to androgens [79]. This protein is commonly found in a TMPRSS2-ERG gene fusion in human prostate carcinomas [79]. This gene fusion results in over expression of ETS transcription factor ERG which plays an important role in progression of CRPC.

1.2.3 AR N/C interaction

The intra-receptor N/C interaction between domains of the AR is classified as a protein-protein interaction needed for transcriptional activity of AR in response to androgens [80]. The biological importance of this interaction is evident in the case of androgen insensitivity syndrome (AIS) [62, 81, 82]. AIS is caused by mutations in the AR LBD that disrupts the N/C interaction resulting in partial or complete failure of masculinization in male phenotype 46XY [62, 81, 82]. This interaction generally involves the ‘^{23}FxxLF^{27}’ motif and/or ‘^{433}WxxLF^{437}’ motifs from AR NTD and the AF2 region in the AR LBD [49, 83]. However in case of interaction with other co-regulatory proteins, a so-called nuclear receptor box ‘LxxLL’ in the co-regulator interacts with the AF2 region of the AR LBD [39, 49, 84, 85]. AR LBD binds the natural ligand DHT and forms a deep hydrophobic groove that could accommodate the bulky

side chain of phenyalanine residues in FxxLF motifs of the AR NTD [55, 56, 86]. Mechanistically, the LxxLL motif in co-regulators (eg, p160 coactivators including SRC1-3) have to compete with the NTD FxxLL motif for binding the same surface on AF2 [39, 49, 84, 85]. However the NTD motif has a 10-fold higher affinity for AF2, hence causes an inhibitory effect on AF2 activity [39, 49, 84, 85]. Through the N/C interaction the transcriptional dominance of the AR is shifted to AF1, marking it as the region responsible for almost all of the receptor transcriptional activity. This further emphasizes the importance of the AR N/C interaction and supports the idea that interaction is preferred to co-activator recruitment in DHT-bound AR [39, 49, 84, 85]. Interestingly, studies have shown that N/C interaction is intra-molecular in the cytoplasm and intermolecular in the nucleus [87]. Once the AR homodimer binds the DNA, the N/C interaction is disrupted and the AF2 pocket, as well as NTD surfaces, becomes available for recruiting co-regulatory proteins [39, 49, 84, 85]. Work by Wilson group has shown that N/C interaction stabilizes the AR by protecting it against degradation and slows down the dissociation rate of bound ligand from the receptor, hence enhancing the receptor activity [80]. As ligand-binding induces AR N/C interaction and results in active transcription of the AR, targeting N/C interaction has also become a unique strategy in AR drug discovery [59, 60, 62].

1.2.4 AR NTD

The AR NTD is unique in sequence with less than 15% amino acid homology to other nuclear hormone receptors [35]. The length of the AR NTD is variable and it is amongst the largest between different family members [88, 89]. The variability in AR NTD is due to the presence of repeat sequences. These repeat regions include 20 to 30 stretches of glutamine, and 16 to 23 stretches of glycine [90-93]. Studies have shown that differences in the length of these

repeat sequences could affect the conformational changes and activity of the receptor and have been associated with genetic diseases [26, 94, 95]. For example, Kennedy's disease or spinal muscular atrophy has been associated with the expansion of the poly-glutamine tract [92]. Work by the Brinkmann and Wilson group has shown that NTD deletion results in transcriptionally silent receptor [39, 43]. They have also highlighted the regions of NTD that are crucial for AR transcriptional activity; known as AF1. AR activation in response to ligand requires the NTD AF-1 (Tau1), which is between residues 101 and 370 with core sequence ^{178}LKDIL^{182} [94, 96]. Tau5 is between residues 360 and 485 with core sequence ^{435}WHTLF^{439}, and is thought to be responsible for activity in the absence of androgen [49]. AF1 is also important for interaction with co-regulatory proteins. Known proteins that directly interact with AF1 include transcription factor IIF subunit 1 (RAP74), steroid receptor co-activator 1-3 (SRC1-3), CREB-binding protein (CBP) /P300, and AR trapped clone 27 (ART27) [97-99].

Contrary to the LBD and DBD, AR NTD is flexible and lacks a stable secondary structure. The AR AF-1 region has less than 13% helical secondary structure [100, 101] and is characterized as a intrinsically disordered protein (IDP) [102]. The importance of IDPs has been recognized only in the past few years. IDPs are major players in cell signaling (70% of proteins) and regulations, and are shown to be involved in pathogenesis of many diseases including cancer [88, 103, 104]. Interestingly, up to 80% of proteins involved in cancers have regions of intrinsic disorder (ID); this includes tumor suppressor P53, c-myc oncogene, and EWS/FLI-1, an important gene fusion in Ewing's sarcomas [105]. Flexibility of IDPs enables them to interact with multiple partners, and these proteins are known to act as hubs. These proteins are known for high specificity but modest affinity in their binding capacities [106-108]. ID regions are also targets for post translational modifications due to their high accessibility [108, 109]. ID regions

of AR are the site of 70% serine phosphorylations [110, 111]. Two residues in the NTD are also known to be modified by sumoylation [112]. Recent studies provide evidence that high net charge and low overall hydrophobicity characterize IDPs and residues D,M, K, R, S, Q, P, and E are known to be disorder promoting [113]. Hence the poly Q tract in the NTD is key for its ID nature and deletion studies of this region have resulted in more structural rigidity of the NTD [44, 90-92, 100, 114].

Work from the McEwan group has shown that AF1 has the structure of molten-globule also referred to as “collapsed disorder” [115]. New models of AF1 suggest that this IDP exists as an ensemble of conformations which depends on the status of the receptor (Figure 3A and 3B) [101, 115-117]. When interacting with coregulatory proteins, bound to DNA, or in the presence of small molecules or a natural osmolyte, AF1 is shown to have higher helical content and becomes more stable. In cellular conditions, interactions with other proteins including members of the general transcriptional machinery are thought to induce folding of AR NTD which is required for its activity [93, 101, 118] (Figure 3A and 3B).

NTD has also been shown to impact intra-domain modulations [119]. For example, NTD causes more specific binding of the receptor to the AREs and reduces non-specific binding to other hormone response elements that have similarities to AREs by modulating the DBD [119]. Despite the importance of AR NTD in receptor activity as mentioned above, no approved drugs targeting this region of the receptor exist to date. Due to the ID nature of NTD, structure-based drug design has been very difficult [96]. However data from the Sadar group has provided proof of concept that NTD is a valid therapeutic target [120]. Decoy molecules of NTD transfected in prostate cancer cells provided the first proof of principle evidence that NTD is a valid target [121]. Decoy molecules resulted in reduction of proliferation of cancer cells and tumors *in vivo*

through mechanisms thought to include competition for key co-regulator proteins. Small molecules and peptide drugs targeting NTD have also been reported by this group which will be discussed later [96, 122, 123].

1.2.5 Ligand independent AR activation in CRPC

The AR NTD contains a number of putative phosphorylation sites for serine-proline-directed kinase, DNA-dependent kinase, protein kinase C, casein kinase I and II, protein kinase A (PKA), mitogen-activated protein kinase (MAPK), Akt, calmodulin kinase II, and tyrosine kinases [63]. Several phosphorylation sites have been identified in the AR; the majority of which map to the AR–NTD [94] particularly the AF-1 region, suggesting that these modifications may directly modulate receptor-dependent transactivation. Based on the characteristics of IDPs, mechanisms of AR NTD activity could involve altering protein–protein interactions and/or changes in NTD structure and stability once bound to DNA and interacting partners.

AR has been shown to be transactivated in the absence of ligand by signaling pathways induced by cytokines such as Interleukin-6 (IL-6) and growth factors such as insulin-like growth factors (IGF-I and IGF-II), and epidermal growth factor (EGF) [124]. The most important methods of ligand independent AR activation perhaps are activation by MAPK, IL6, and PKA. MAPK and PKA pathways are shown to involve transactivation of NTD in the absence of ligand. MAPK is elevated in recurrent prostate cancer [125] and required for both ligand-dependent and ligand-independent activation of the AR [126]. Androgen, IL-6 and stimulation of the PKA pathway all increase MAPK phosphorylation [126, 127]. Consequently, MAPK phorphorylates and activates the AR resulting in expression of AR target genes, recruitment of co-regulatory proteins, and proliferation of prostate cancer cells [128]. Phosphorylation of AR by MAPK could

also result in hyper-sensitization of the receptor to low levels of androgens in LNCaP cells [129]. This alternate pathway of activation is one of the mechanisms of resistance to castration and re-activation of the AR in CRPC.

AR is also activated by IL-6 in the presence of functional JAKs/STAT3 and MAPK pathways [130, 131]. Immunoprecipitation studies have found formation of a complex between amino acids 234–558 of the AR-NTD and STAT3 as a result of IL-6 treatment in LNCaP cells [126]. Importantly, CBP and P300, which are key cofactors for AR transcriptional activity, are also involved downstream of the MAPK pathway during transactivation of the AR by IL-6 [132]. Clinical studies with antibodies targeting IL6 however, have shown no significant benefit, although IL-6 levels are shown to be significantly increased in the serum and bone metastases of patients with CRPC [133, 134].

Ligand-independent activation of the AR by the PKA pathway caused enhanced AR-ARE complex formation compared to the ligand dependent activated AR, suggesting the importance of this mode of AR activation. PKA interaction with the AR was shown in human prostate cancer cells following androgen deprivation [135-137]. AR NTD activation by PKA in LNCaP cells also enhances the stability of the receptor-DNA complex which is important for transcriptional activity of the AR [135, 138].

Activation of AR through the NTD by these non canonical mechanisms suggests that NTD plays an important role in AR signaling in all stages of prostate cancer including CRPC and highlights this IDP as a valid target for inhibition of the receptor activity.

1.3 Pathology of CRPC

1.3.1 AR amplification and/or over-expression

AR is upregulated in CRPC and is a well-known and critical factor in progression of CRPC. Studies have shown that AR is expressed at both transcript and protein level in the majority of prostate cancers with the exception of neuroendocrine tumors [33, 139]. Gene expression studies have demonstrated that AR is the most common up-regulated gene in response to androgen deprivation [140-147]. Comprehensive studies with large sample sizes confirmed that the most frequent somatic AR alteration in prostate cancer progression has been gene amplification [140, 148-150]. A study by Chen et al. demonstrated that AR overexpression was both necessary and sufficient for progression of the prostate cancer from androgen dependent to CRPC in xenografts [151]. Other studies have also shown that AR overexpression was sufficient for tumor formation in castrated SCID mice [151]. AR overexpression alters the coregulators that are recruited to the AR promoter and helps the conversion of antiandrogens such as bicalutamide and flutamide from antagonists to agonists [151-154]. In a LNCaP cell line model over expressing the wild-type AR, binding of AR to the chromatin was shown to be sensitized and the level of androgen regulated genes increased by 3- to 5-fold, emphasizing the sensitivity of the cells to the low androgen levels [155]. In clinical CRPC tumors, AR was shown to be nuclear, hence believed to be transcriptionally active, regardless of the serum androgen levels. AR gene copy number increase is present in about 80% of CRPC, but AR protein overexpression is present in approximately 30% of clinical CRPC specimens [145, 156, 157]. Of note is that this effect is rarely found in primary tumors prior to treatment, suggesting that AR amplification is a result of a selection process following ADT [157].

Mechanisms of AR overexpression without gene amplification are currently under investigation. Intriguingly, recent studies have revealed links to regulation of AR expression by microRNAs [38], through retinoblastoma protein (RB1) [158] and/or auto-regulation of AR mRNA [159-161], all contributing to over expression of AR in CRPC.

1.3.2 Alterations in AR co-regulators

AR is involved in multiple biological processes and interacts with at least 169 proteins [72, 97, 162]. AR interacting proteins include families of proteins involved in chromatin remodeling, histone modifying, the sumoylation pathway, splicing and RNA metabolism, DNA repair, chaperones, cytoskeletal proteins, signal integrators and transducers, scaffolds and adaptors, and cell cycle and apoptosis regulators [97]. Among these families, proteins with an impact on AR transcriptional activity are referred to as co-regulatory proteins which include the co-activators and co-repressors. Increased expression of co-activators such as SRC1-3, CBP/P300, PRK1, MAGE-11, TIF-1, SRB-1 and NFκB or decreased expression of co-repressors such as the nuclear receptor corepressor (NCoR), and the silencing mediator of retinoic acid and thyroid hormone receptor (SMRT) are proposed mechanisms that sensitize the AR to low androgen conditions or convert antagonists into AR agonists [97, 163, 164]. Hence alteration in expression of co-regulators is one of the hallmarks of CRPC. Based on these observations, developing therapies to target AR co-regulators through disruption of protein-protein interactions has been proposed for inhibiting AR activity in CRPC [29, 165].

1.3.3 AR mutations

About 75% of prostate cancer tumors carry TMPRSS2-ERG gene fusions [166], but the overall mutation rates are relatively low in prostate cancer compared to other cancers. Mutations

of AR have frequently been identified, mostly in response to androgen deprivation and many are gain of function mutations [167-172]. In some tumors, these mutations could sensitize AR to low androgen levels and alter ligand specificity, all known to be part of the mechanisms of resistance to therapy [69]. In studies of metastatic samples, approximately 44% of hormone-naïve cases showed a mutation in the AR, 50% of which were in the NTD with no known effect on the receptor function [173]. An interesting question would be whether mutations in the AR NTD would occur following treatment with NTD inhibitors such as EPI-001, and what, if any, would the effect be of such mutations. As NTD inhibitors have recently been introduced [122] such studies are awaited. However, AR mutations in the ligand-binding domain (LBD) that result in creation of promiscuous receptors have been described. The most important mutation in prostate cancer which was first identified in the LNCaP prostate cancer cell line, and later in several advanced prostate cancers is T877A [169]. This mutation alters the helix 11 and LBD structure; hence interaction of the receptor with the ligand is altered. Due to the T877A mutation, specificity of LBD is broadened which allows various molecules including the antiandrogen hydroxyflutamide, progestins, estradiol, and corticosteroids to act as AR agonists and change the receptor to an active conformation [174]. Other important AR mutations include H874Y and W741C. The H874Y mutation was identified in patients treated with flutamide [175]. This mutation causes a change in the conformation of the receptor and impairs the interaction with the p160 coactivator proteins and alters ligand specificity [176, 177]. W741C mutation is mostly found in bicalutamide treated patients [171, 178], and has been shown to play a role in conversion of the antiandrogens from antagonist to agonist. Sequencing studies have identified promiscuous AR mutants, including T877A in only 5–10% of the clinical patient samples [140, 150]; however, as evidence suggests that commonly administrated antiandrogens have potential

agonist activity on AR mutations or over/expression settings [179], the need for novel AR antagonists without agonist activity is valid and critical.

Consistent with the above, it is observed that AR mutations are identified post ADT in approximately 20% to 40% of CRPC tumors, but rarely found in hormone-naïve prostate tumors [33]. Therefore, a need for development of therapeutic agents with better target specificity and binding affinity to potentially avoid the selection for AR mutations is also valid [170, 180].

1.3.4 AR splice variants

Recent discovery of splice variants of the AR from human tissues and prostate cancer cell lines has been a significant breakthrough in the prostate cancer field [181-185]. Alternative splicing occurs in about 90% of human genes [186, 187], and IDP are known to have high frequency of splice variants [188, 189]. Approximately, 25 AR splice variants have been identified, many of which identified in the 22Rv1 cell line [29]. Expression of AR variant transcripts has been confirmed in VCaP and LNCaP95 cell lines, LuCaP xenografts, clinical CRPC specimen and metastases samples [181-185]. However, detection of variants at the protein level has proved to be difficult due to lack of specific antibodies to each variant [139]. To date the only AR variant detected at the protein level with a specific antibody is AR3/ARV7 [183, 185]. Although many of the variants have no known function, some show constitutive activity [190]. Interestingly, deletion studies of the AR LBD have been shown to result in a constitutively active AR, suggesting that LBD has a repressing effect on the NTD transcriptional activity [40]. Consistent with this observation, some of the AR splice variants lacking the LBD are shown to have constitutive activity [182]. Two clinically relevant AR variants, commonly found in human CRPC samples, are AR3/ARV7 and AR^{v567es} [182, 183, 191]. AR3/ARV7 has an intact NTD and

DBD, but lacks the hinge region and the LBD and has 16 unique amino acids after the DBD. AR^{v567es} has the NTD, DBD and a part of the hinge region, but exons 5, 6, and 7 are excluded causing a frame shift and a truncated receptor with 10 novel peptides from exons 4 and 8 [139]. Both ARV7 and AR^{v567es} which appear to have lost the AR LBD show ligand-independent constitutive activity, emphasizing the critical role of an intact NTD in these variants [182, 192].

Immunohistochemistry studies by Guo et al has demonstrated that AR3/ARV7 is upregulated in CRPC samples, and the levels of this variant correlate with risk of tumor recurrence following radical prostatectomy [184]. Studies have shown that transfection of AR^{v567es} in cells yields tumors that are resistant to castration emphasizing the biological and functional importance of this splice variant [182]. In addition, in clinical samples, examination of 69 metastases from men who died of CRPC showed that out of 46 samples that expressed full-length AR or variant, 80% expressed full-length AR, 73% expressed AR^{v567es} and/or AR3/ARV7, and as much as 20% of metastases expressed only AR^{v567es} [139, 182]. This observation further emphasizes the importance of AR^{v567es} expression and function in patient response to drugs. This observation also suggests that current therapies that target the AR LBD, including antiandrogens bicalutamide and MDV3100 would have no effect on the metastases that solely express variants [193]. The underlying cause of expression of AR variants is unknown and currently under investigation. Given the frequency of AR3/ARV7 and AR^{v567es} expression in clinical CRPC samples, and consistent increase in expression of the same variants following androgen deprivation *in vitro*, it is suggested that ADT could select for the tumors that express these variants [25, 194, 195]. To date, known mechanisms of variant expression are intragenic rearrangements and DNA deletion events [196, 197]. However, misregulation of the splicing events could not be ruled out [198], and more studies are required for a comprehensive

understanding of the mechanisms of expression of these species. The splice variants of the AR regulate a distinct but overlapping set of target genes as shown by microarray analysis [182, 185]. UBE2C and AKT1 are examples of genes that are regulated by AR^{v567es} and AR3/ARV7 respectively, suggesting a role for AR variants in regulating cell cycle gene expression [182, 184, 199].

The interaction between the full-length AR and the variants has also been studied [181, 182]. Theoretically, all nuclear AR variants could have transcriptional activity independent of the full-length AR. Both AR^{v567es} and AR3/ARV7 have constitutive activity on their own when transfected in AR negative cell lines [179, 181]. However, in a study by the Plymate group, full-length AR has been found in a complex with AR^{v567es}, and this complex increases AR levels and activity by preventing degradation of full-length AR [182]. The authors also show that AR^{v567es} can cause nuclear translocation of the full-length receptor in the absence of androgens. AR3/ARV7, on the other hand, has not been found in a complex with the full-length AR [182]. However, a study by Watson et al showed that antiandrogen MDV3100 could block the growth of CRPC tumors expressing both AR3/ARV7 and full-length AR, suggesting a dependency of variant activity on the full-length receptor [181]. Effect of MDV3100 on AR^{v567es} activity in presence or absence of full-length AR has not been reported. Of note is the ratio of expression of full-length AR to variants which seems to be an important indicator of tumor response to castration [182], and must be considered when investigating the effect of drugs on these variants. Taken together, data suggests that at least some AR variants could have activity in the absence of full-length AR, and the current antiandrogen therapies would not be effective in targeting these cases. Novel therapies targeting the variants directly, through the NTD, should have immediate clinical benefit for patients [120].

1.3.5 Ligand-dependent AR activation in CRPC

Recent evidence suggests that androgens are not eliminated from the tumor microenvironment despite castration. Tissue androgen measurements in men with locally recurrent or metastatic CRPC demonstrate that levels of residual androgen are sufficient for continuous AR signaling and AR-mediated gene expression [25, 200, 201]. Alterations in several critical enzymes have been shown to cause intracrine androgen production and maintain the tumor androgen microenvironment in CRPC [202]. Two important mechanisms of androgen synthesis in CRPC tumors have been described from studying the prostate tumor samples from castrate patients: 1) alterations in key enzymes responsible for steroid biosynthesis and androgen metabolism resulting in the *de novo* synthesis of androgens from precursors such as progestins and cholesterol [203-205], and 2) detection of adrenal androgens at significant levels in the prostate tissue of castrate men [206]. These other sources of androgens are speculated to facilitate the continuous AR signaling in CRPC [29], and highlight the androgen synthesis pathways as therapeutic targets for drug development.

1.4 Novel therapies for CRPC

Prior to 2010, the final treatment option for patients with advanced prostate cancer following ADT failure was chemotherapy using agents such as mitoxantrone and docetaxel with only better quality of life in case of mitoxantrone and survival benefits of only a few months for docetaxel. Since 2010, five new treatments for CRPC have been approved which include cabazitaxel, sipuleucel-T, and abiraterone acetate [30, 207, 208]. These novel therapies have given patients hope of living longer with improved quality of life. However, it is still critical to understand the molecular mechanism of drug activity and resistance, for the purpose of

development of the next class of drugs to meet the critical need of novel therapeutics in these patients.

1.4.1 Non-hormonal: chemotherapy- docetaxel and cabazitaxel

Mitoxantrone plus prednisone was the standard chemotherapy treatment for advanced prostate cancer prior to 2004 resulting in reduced pain and better quality of life, however this treatment did not improve survival in these patients [209, 210]. Docetaxel chemotherapy was approved for treatment of patients with CRPC following promising results in a clinical trial in 2004. In this trial, the docetaxel plus prednisone group had a median survival of 18.9 months compared to16.5 months in the mitoxantrone group. Up to 50% of patients in the docetaxel group also had at least 50% reduction in the PSA levels, and 20% had improved quality of life [211].

Cabazitaxel is a novel tubulin-binding chemotherapy agent approved by FDA for treatment of CRPC patients with tumors resistant to docetaxel therapy. In the clinical trial of patients which CRPC that progressed despite docetaxel therapy, the cabazitaxel plus prednisone group showed 15.1 month survival compared to 12.7 months in the mitoxantrone group [30, 209].

1.4.2 Non-hormonal: immunotherapy and sipuleucel-T

Recently a novel therapy for metastatic castration resistant prostate cancer was approved by FDA. Sipuleucel-T (Provenge®, Dendreon, Seattle, WA, USA) is an active immunotherapy agent that stimulates the patient's immune system to mount an attack against the cancer cells but is not toxic for surrounding tissues. To initiate an efficacious immune response, sipuleucel-T includes an autologous antigen-presenting cell vaccine loaded with prostate acid phosphatase conjugated with granulocyte-macrophage colony-stimulating factor (GM-CSF) [212]. A double-

blind, placebo-controlled trial, called the Immunotherapy for Prostate Adenocarcinoma Treatment (IMPACT) trial [213] assessed sipuleucel-T and concluded that this treatment yielded a survival advantage in men with metastatic castration-resistant prostate cancer. This trial reported a 22% reduction in the risk of death represented by a 4.1 month improvement in median survival in the treatment group compared to the placebo control (25.8 months in the sipuleucel-T group vs. 21.7 months in the placebo group). However, an intensely debated issue is the cost of sipuleucel-T therapy. Considering that the cost of sipuleucel-T is about an estimated $93,000 per patient for three doses, the financial burden of this therapy might seem high compared with the traditional chemotherapy agents such as docetaxel [207].

1.4.3 Hormonal: CYP17A inhibitors- abiraterone acetate

As CRPC tumors are shown to be AR-dependent, therapeutic strategies have been focused on targeting residual tumoral androgens more effectively. Alterations in steroid enzymes, responsible for androgen metabolism, synthesis or conversion are known to be a mechanism of CRPC development [214]. These alterations potentiate the use of adrenal androgens to synthesize testosterone and DHT and/or inhibit the conversion of DHT to inactive metabolites. Alterations in steroid metabolism within the tumor are also reported and are believed to facilitate tumor development [194]. Hence, enzymes in steriod metabolic pathways are valid therapeutic targets for CRPC drug development.

An important enzyme responsible for catalyzing the steps in the conversion of progesterone precursors to the adrenal androgens and dehydroepiandrosterone (DHEA) is CYP17A. Several potent CYP17A inhibitors have been reported, and abiraterone acetate (abiraterone) is the recent agent that was approved by FDA for treating CRPC patients [215].

Abiraterone is a selective irreversible inhibitor of CYP17 with efficacy in achieving PSA reduction in CRPC patients. In castrate men, abiraterone showed about 75% or higher reduction in serum testosterone levels [216]. Clinical trials of abiraterone have demonstrated survival benefits. Phase 1/2 studies demonstrated PSA reduction of at least 50% in approximately two-thirds of patients with metastatic CRPC pre-chemotherapy [217, 218]. In the phase 3 study, in the post-chemotherapy patients, after a median follow-up of 12.8 months, the abiraterone group had a survival benefit of 14.8 months vs. the placebo group of 10.9 months showing a 35% reduction in risk of death [219]. Results exceeded the study criteria, and the study was terminated with the patients in the placebo arm given treatment.

Mechanisms of resistance to abiraterone acetate are currently under intense investigation. A study by Mostaghel et al. has shown that further reduction of prostate tissue levels of androgen by CYP17 inhibitors may result in elevated levels of constitutively active AR splice variant in patients post abiraterone treatment [220]. These variant species are thought to be a cause of the eventual treatment failure and the difficulty of developing therapies for patients following abiraterone failure [220].

1.4.4 Hormonal: antiandrogens- bicalutamide

Antiandrogens have been used for the treatment of prostate diseases for many years, and are also used for the treatment of male baldness and acne due to excessive androgen stimulation. Two classes of AR antiandrogens are used in the clinic which include steroidal and nonsteroidal antiandrogens. The naming refers to the structural characteristics of these agents. Steroidal compounds are similar to the natural ligand DHT with 4 rings and planar structure while nonsteroidal antiandrogens have distinct pharmacophores structurally [221]. Nonsteroidal

antiandrogens show an improved oral bioavailability [222], hence have better delivery for the patients. The first nonsteroidal antiandrogen, flutamide, was approved for treatment of prostate cancer in 1989, and related compounds bicalutamide and nilutamide were approved later in 1995 [223, 224]. Antiandrogens directly block androgen binding to AR, regardless of the source of the hormone, either testicular or adrenal as they compete with the ligand for the same binding pocket. Antiandrogens could be used as a first line therapy alone; however they are usually administered with GnRH analoges for complete androgen blockade [225]. This is due to a compensation mechanism following administration of antiandrogens, an increased luteinizing hormone [LH] release to keep up with testosterone production [23]. Higher LH production also potentiates the excess production of active metabolite 17β-estradiol which causes the common side effect of antiandrogen treatment gynecomastia (enlargement of breasts in males) [23]. GnRH analogs target the endogenous testosterone production axis; hence enhancing the effect of antiandrogens, and resulting in a more complete androgen blockage. Although antiandrogens show an initial response in majority of patients, resistance to antiandrogens is common, as seen in antiandrogen withdrawal syndrome (AWS) [226, 227]. In AWS, patients often present with regression of tumors if antiandrogen treatment is ceased. This antiandrogen failure is shown to be due to emergence of promiscuous AR, including the mutants with broad specificity for ligand [228, 229]. AR mutations, including T877A and W741C, are of particular interest as they result in agonist activity of the antiandrogens [179, 226, 227, 230]. Consistent with the results from previous sections, an urgent need for development of AR antiandrogen with high degree of target specificity and affinity still exists [23, 231]. A novel and promising antiandrogen currently in clinical trials for CRPC is MDV3100.

1.4.5 Hormonal: antiandrogens- MDV3100

MDV3100 is a second-generation antiandrogen that binds to the AR with up to fine to eight-fold greater affinity than the classic antiandrogens like bicalutamide. Studies have shown that, the nuclear translocation of AR is significantly lower in MDV3100 treatment compared to bicalutamide [57, 232]. ChIP studies show lower chromatin occupancy at AREs, and reduction in proliferation of LNCaP prostate cancer cells is also reported as a result of MDV3100 treatment [57]. AR is amplified in about 20% to 25% of CRPC cases [145]. This population of patients may be the niche that would benefit the most from treatment with MDV3100 based on the potency and specificity of this anti-androgen. However, MDV3100 inhibition could be overcome by addition of androgens, suggesting that MDV3100 may not be equally effective in cases of nonamplified AR or tumors with residual tumor androgens [32]. The effect of MDV3100 on AR splice variants is dependent on the presence of full-length AR and the interaction between the two species [181]. In metastatic CRPC samples with AR variants being the solely expressed transcript, MDV3100 is expected to have no therapeutic benefits. Xenograft models using human tumors that naturally express AR^{v567es}, such as LuCaP 86.2, would provide a better and more relevant model for assessing the effects of MDV3100 and other AR antagonists on CRPC tumors expressing AR variants [182].

The clinical effect of MDV3100 was assessed in a phase 1/2 trial with 140 patients with progressive, metastatic, castration-resistant prostate cancer. This trial included dose-escalation (30 to 600 mg) cohorts with three to six patients given an oral daily dose of MDV3100 [233]. Importantly, 62% of chemotherapy-naïve patients showed 50% reduction in PSA [233]. All patients who were assessed by PET imaging tomography scans showed declines in accumulation of tumors in soft tissue and bone. A phase 3, randomized, placebo controlled trial of MDV3100

in docetaxel-treated men with metastatic CRPC recently concluded with 4.8 month survival benefit for the treatment group. Based on the monitoring committee recommendation the study ended early and the treatment was offered to the placebo group. Further analysis of data from this trial is awaited [30, 207].

Promising data from these trials has led to investigation of MDV3100 in other trials. Two ongoing MDV3100 trials are assessing the effect of this drug at earlier stages of the disease. One is the first line treatment in men with prostate cancer prior to ADT and another a replacement for antiandrogen bicalutamide for ADT in combination with LHRH agonist/antagonist [30]. Results of such trials will further elucidate the most beneficial time and stage of the disease that MDV3100 could be administered to the patients.

1.5 Intrinsically disordered proteins as targets for drug development

Membrane receptors and enzymes represent the majority of drug targets mostly due to structural data available for enzyme-substrate and receptor-ligand interactions [234]. These rigid structures are used for rational drug design that aims to identify drugs that have high affinity for binding the active site of the enzyme or binding the receptor as an agonist. However, in many diseases such as cancer, failed protein-protein interactions are also known as important causes of disease progression [235]. These protein-protein interactions have a significant potential as targets for drug development, but a main challenge is the identification of the binding surface of these proteins [236]. In many instances, the large surfaces of protein interactions include smaller key residues (known as “hot spots”) that are crucial for interaction [237, 238], and once identified can be targeted by small molecule drugs. Key players in the context of protein-protein interactions are the IDPs. IDPs play important roles in biological systems [105]. Well known

tumor suppressors including p53 and oncogenes such as c-myc, possess intrinsically disordered regions [239-241], underlining this structure as a requirement for the function of these proteins. The flexible nature of IDP, which enables them to interact with multiple partner proteins, makes these common and essential players of biological functions also desirable targets for small molecule drug design [104, 241, 242].

1.5.1 Small molecule drugs for IDPs

Two approaches can be considered in designing small molecule inhibitors of IDPs with focus on blocking protein-protein interactions [243]. The first approach is based on a binding protein with a known structure, and the IDP that undergoes disorder to order conformation upon binding this partner. This method allows for recognition of small regions of the IDP that are key for interaction, when IDPs are in complex with the partner protein. This binding is then mimicked by the small molecule inhibitor in structure based rational drug design. In the other approach, one or both binding partners could be ID, and no high-resolution structural data from the binding partner is required. Small molecule drugs are usually identified by screening libraries of compounds that directly bind the hot spots on the IDPs [244]. This binding changes the overall disorder state of the ID region to a more stable conformation. This step replaces the need for the partner protein binding to create the stabilized state and blocks protein-protein interactions [245, 246].

Metallo and colleagues have identified seven structurally unrelated small molecule inhibitors for the ID regions of c-myc oncoprotein by screening libraries [239-241]. They have further identified 3 hot spots that each consist of about 10 residues in the ID regions of c-myc and demonstrated that all seven inhibitors bind one of these three regions [241, 247].

Interestingly, binding of one inhibitor does not affect binding of others, and these inhibitors can bind independently and simultaneously [240, 241].

Our approach to drug design for the ID AR NTD was based on a similar rationale as of the c-myc drug discovery studies. However, an important awaiting study in the studies of AR NTD is identification of hot spots in the AF1 region of NTD which is still under investigation. Two classes of inhibitors of the AR NTD have been identified based on this approach, using high throughput screening of libraries of sponge extracts. First is EPI-001, a small molecule by product of bisphenol A. EPI-001 shows promising pre-clinical evidence for inhibiting AR activity *in vitro* and in CRPC xenografts in mice [122]. Second are sintokamides, chlorinated peptides isolated from the marine sponge *Dysidea* species which also inhibit transactivation of AR NTD (Figure 4). In this project, we aim to further characterize this group of compounds [122, 123]. To provide the proof of principle that such hot spots do exist in AR NTD and drugs could target each site independent of others, we also investigated the possibility of combination therapies with the existing NTD inhibitors. Through this indirect approach, an additive inhibitory effect would be an indication that the two structurally unrelated compounds possibly interact with different hot spots on the AR NTD. Consistent with the c-myc inhibitor design studies [240, 241], these compounds could be targeting the NTD independently and simultaneously.

1.5.2 AR NTD as a target for therapy in CRPC

Deletion experiments have shown that the AR NTD is essential for transcriptional activity of the AR by both ligand dependent and independent mechanisms [40]. Antiandrogens such as MDV3100 that target the AR LBD are efficient in inhibiting the CRPC cases with over expression of full-length AR, however these agents may be ineffective in targeting the following

mechanisms of CRPC 1) Inhibiting ligand-independent transactivation of the AR NTD by IL-6 and other growth factors as this occurs independent of ligand and LBD functions [63] 2) Inhibiting the constitutively active AR splice variants lacking the LBD [139]. Studies have demonstrated that MDV3100 inhibited the ARV7 activity in the presence of full-length receptor, however this drug had no effect on inhibition of this variant independent of the full-length receptor [181].

An alternative approach to targeting the AR LBD is developing inhibitors to the NTD. Our group provided the first *in vivo* proof-of-principle that NTD is a valid therapeutic target. This was demonstrated by employing decoy molecules encoding residues 1 to 558 of the AR NTD [121]. AR NTD decoy molecules inhibited AR activity and blocked both androgen-dependent and CRPC tumor growth. Although the mechanism of decoy activity is under investigation, a possible mechanism is the competition of the decoys with the full-length receptor for the key co-regulatory proteins essential for AR transcriptional activity [121]. Due to the large size of the AR NTD and possibility of fast peptides metabolism, systemic delivery of AR NTD decoys in a clinical setting could be a significant challenge. Our group employed high throughput screening methods to find small molecule inhibitors for the AR NTD from sponges [193]. We employed a new assay to screen a library of marine natural product extracts for molecules that inhibit transactivation of the AR NTD in LNCaP human prostate cancer cells. The cells stably expressing the ARR3-luciferase reporter were treated with marine sponge extracts, and luciferase activity was assessed as a measure of drug potency. The goal for developing such small molecule inhibitors is to overcome the difficulties of therapeutic development of AR NTD decoys [120, 121], and hence effectively target the NTD to suppress AR activity.

1.5.3 Pharmacokinetics

Promising preclinical data must be proven beneficial in order to move to treating patients in clinical trials. Statistically, up to 40% of new drugs that reach the clinical stage fail due to undesirable pharmacokinetics and toxicity [248]. Important pharmacokinetics properties include half-life ($t_{1/2}$) and bioavailability [249]. The $t_{1/2}$ is the period of time it takes for the concentration of drug in the blood to be reduced to one-half. In drug development, the amount of drug in plasma is considered which indicates how fast a drug is eliminated from the plasma. The importance of $t_{1/2}$ of drugs is based on the determination of the dose and intervals required to achieve a therapeutic effect in patients. If the drug has a very short $t_{1/2}$, frequent dosing would be required. However the $t_{1/2}$ of drugs can be modified by changing the chemical structure of the compounds. Changes in chemical structure could result in a decrease in the clearance of drug from plasma or increase its volume of distribution (volume of a drug needed to reach a certain concentration in the blood, considering distribution to the other tissues).

The oral bioavailability of a drug refers to the percentage of the oral dose of the drug that reaches the blood stream after oral administration. The metabolism and absorption of a compound are important factors that affect bioavailability. Many physiological factors influence the absorption of a drug. The epithelial cells covering the walls of gastrointestinal (GI) tract have compact phospholipid membranes [250]; hence drugs must possess properties such as lipophilicity to penetrate this membrane [251]. Generally, drugs with poor lipophilicity will not be absorbed efficiently by oral administration [252]. In addition, the blood supply of the GI tract must pass through the liver before the systemic circulation, hence a high chance of metabolic breakdown of compounds exists which further complicates the absorption and lowers the drug

bioavailability [253]. These factors must be studied to determine the most efficient means of administration of drugs [251].

Two classes of drug are considered here as inhibitors of AR NTD that include peptides and small molecules [122, 123]. Both groups have advantages and disadvantages hence pharmacokinetics studies are necessary to confirm their qualification for a drug candidate. Peptides and small molecules drugs have been studied for many years in drug development and therapeutics fields [251]. In general, small molecules have the virtues of small size and membrane permeability, easier chemical synthesis, low price, and high oral availability. The peptides are commonly larger, more expensive to synthesize, have faster clearance from circulations and are less stable [251, 254]. Hence, peptides usually need to be injected rather than administered orally. However, they show higher specificity, few toxicology problems, more potency, less accumulation in organs, and less drug-drug interaction challenges [255]. The peptide inhibitors studied in this thesis (sintokamides) have a smaller size and a unique side chain, making them candidates with potential properties from both groups. Fortunately, modern synthetic chemistry enables modifications to the structure of compounds that could optimize factors such as bioavailability and half-life (discussed later).

1.5.4 Target specificity

An essential step in characterization of novel inhibitors is ruling out off target and potentially toxic side effects of drugs. The toxicity could initiate from inhibition of target in tissues other than the specific targeted tissue or inhibition of a common molecular target, such as members of the general transcription or translation machinery. Hence it is essential to characterize the specificity of novel AR antagonists. AR is known to be highly expressed in

tissues such as the epididymis, prostate, vas deferens, adrenal gland, kidney and skeletal muscle [256, 257]. AR antagonists (referring to the commonly used non-steroidal antiandrogens) are relatively well tolerated, demonstrating that targeting AR in these tissues is not associated with severe adverse effects. At the molecular level, the most highly related proteins to AR in the human body include other members of the human steroid receptors, progesterone receptor (PR) and glucocorticoid receptor (GR) [94]. AR has higher than 70% amino acids sequence similarities with PR and GR in the DNA-binding domain (DBD). Although the AR NTD shares less than 15% homology with the NTDs of PR and GR [26, 44], overall they share common transcriptional co-regulators including the members of the basal transcriptional machinery such as coactivator SRC1 and corepressors NCoR and SMRT [94].

Inhibition of PR is thought to have a minimal effect on prostate cancer patients, as this receptor activity is predominantly important in females. However, inhibition of PR could broaden the implication and value of AR inhibitors. Studies have shown that PR agonist/antagonists can be used for treating menorrhagia and leiomyoma tumors [258]. These inhibitors also have the potential for treatment of endometriosis and hormone-dependent cancers [258].

GR, on the other hand, is highly expressed in almost all major physiological systems including the central nervous system (CNS), endocrine, metabolic, gastrointestinal, immune, reproductive, cardiovascular, and respiratory systems. GR plays an important role in a variety of important developmental and physiological processes. Regulation of carbohydrate metabolism and inflammatory responses are well known functions of GR [259, 260]. GR is a major therapeutic target for a variety of anti-inflammatory drugs, however long term inhibition of GR

could cause severe side effects such as osteoporosis, water retention, diabetes and psychosis [261].

1.5.5 EPI-001

At present the most promising published compound targeting the AR NTD is EPI-001. This compound is a metabolite of bisphenyl A which was isolated from marine sponge *Geodia lindgreni* by a high throughput screening assay. EPI-001 shows strong evidence for inhibition of AR in both *in vitro* and *in vivo* studies [122].

In summary, EPI-001: inhibits AR activity induced by androgen, forskolin (which stimulates PKA activity) and IL-6; does not bind the AR LBD; does not cause nuclear translocation of the AR; blocks AR-dependent proliferation of LNCaP cells; inhibits AR interaction with CBP and RAP74; inhibits N/C interaction of the AR; inhibits androgen-induced expression of PSA and TMPRSS2 androgen-responsive genes, and reduces AR-ARE interaction [122]. Sadar et al unpublished data show that as predicted, EPI-001 inhibits the transcriptional activity of the naturally occurring splice variants ARV7 and AR^{v567es} *in vitro*. Hence, it is the first inhibitor available for direct targeting of tumors that solely express variants [182].

Importantly, EPI-001 caused tumor regression of CRPC when administered by i.v. injection after 2 weeks of treatment [122]. No toxicity was observed in animals demonstrated from animal body weight and histology of the organs [122]. Pharmacokinetic studies show that EPI-001 has 86% oral bioavailability.

EPI-001 is a mixture of 4 stereoisomers. Preclinical development of EPI-001 revealed that EPI-002, a single stereoisomer (2R, 20S) showed improved properties compared to other

stereoisomers or the mixture. Analogues of EPI-001 have been characterized, and one has been selected as a clinical candidate and is expected to be tested in clinical trials in 2013.

1.5.6 Discovery, isolation and structure of sintokamide A

Sintokamides are a class of compounds unrelated to EPI-001 which were also found by a high throughput screening assay as novel antagonists of AR NTD. Sintokamides are natural compounds with a peptide structure. Isolation and discovery of sintokamide structures have been reported [123].

Specimens of *Dysidea sp*. were collected near Palau Sintok, Karimunjawa archipelago, Indonesia. The sponge specimens were extracted and fractionated. Chromatography and reversed-phase HPLC techniques resulted in pure samples of sintokamides A to E. Although these chlorinated peptides have been isolated from marine sponges they also have features of cyanobacterial metabolism, suggesting a microbial origin for the compounds. Sintokamide A (SINT1) has a molecular formula of $C_{18}H_{25}N_2O_4Cl_5$ and absolute configuration 2S, 4S, 10R, 16S (Figure 4). The structure of this compound was confirmed by nuclear magnetic resonance spectroscopy and crystals that were characterized by X-ray diffraction analysis [123]. Structural activity relationships demonstrated that the chlorine groups enhance the activity of this class of compounds (Sadar and Andersen unpublished results), and the total number of chlorines was a determining factor in selection of SINT1 as a candidate for biological studies.

SINT1 was then assessed in biological assays for efficacy and safety. SINT1 blocked AR activity as measured using the PSA (6.1)-luciferase reporter in LNCaP prostate cancer cells. SINT1 was also effective in blocking androgen-induced proliferation in androgen-sensitive LNCaP prostate cancer cells but not PC3 cells that do not express AR and do not rely on the AR

for growth and survival. The morphology of LNCaP cells treated with SINT1 showed no obvious signs of toxicity, indicating no general cytotoxicity and specificity of the compound [123]. To determine if SINT1 blocked transactivation of the AR NTD, the AR NTD-Gal4DBD chimera protein was transfected in LNCaP cells and induced by forskolin (FSK), to activate the NTD through the PKA pathway. SINT1 reduced FSK induced transactivation of the AR NTD to baseline levels. In this project, we aim to further characterize SINT1 and assess its activity in inhibition of AR activity and investigate its mechanism of action.

1.6 Summary and research objectives

1.6.1 Objectives and hypothesis

Currently there is no cure for advanced prostate cancer. Recent evidence has revealed the continuous importance of androgens and AR in progression of the disease. Novel therapies targeting this axis including abiraterone acetate, inhibitor of androgen biosynthesis, and MDV3100, an antiandrogen that potently binds AR, have shown clinical benefits for treatment of the disease. However, these therapies eventually fail through mechanisms that may include expression of constitutively active AR splice variants lacking the AR LBD. Hence inhibitors of the AR NTD are the only compounds that could directly target the AR variants and be used for patients with metastatic lesions that solely express these variants. With introduction of EPI-001, the AR NTD has been shown to be a valid drug target for CRPC. EPI-001 inhibits the transactivation of NTD and has very promising *in vivo* efficacy in blocking the growth of CRPC xenografts. Other novel compounds targeting the NTD are now being developed in hope of better efficacy by passing possible resistance mechanisms, and promise of combination therapies that would have better clinical benefits for the patients. SINT1 is an unrelated class of molecules

that was identified by high throughput screening of libraries from marine sponge extracts. We hypothesize that SINT1 is an antagonist of the AR NTD. We aim to provide proof-of-principle evidence that blocking the AR NTD by SINT1 is an efficacious way to inhibit activity of the AR *in vitro* and block growth of CRPC xenografts in mice.

1.6.2 Objectives

The objectives of this thesis were the following:

-To evaluate the specificity of SINT1 for AR

-To determine if SINT1 blocks transcriptional activity of the AR

-To measure effect of SINT1 on endogenous expression of androgen-responsive PSA gene by qRT PCR analysis

-To elucidate the mechanism of SINT1 inhibition of AR transactivation by assessing AR N/C interaction, AR nuclear translocation, and inhibition of ligand binding

-To investigate the effect of SINT1 combination with EPI-002 on AR transactivation

- To assess the pharmacokinetics parameters of SINT1

-To measure SINT1 efficacy *in vivo* in LNCaP xenografts in castrate mice

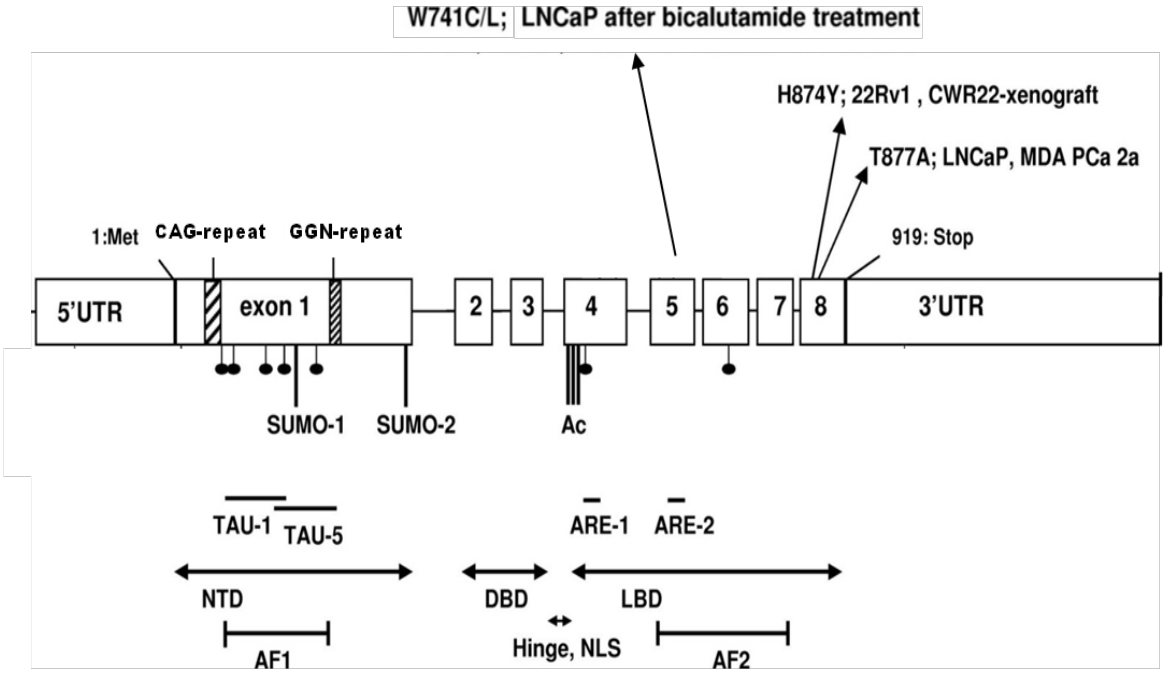

Figure 1. The structure of the AR.

Functional regions are illustrated under the structure of AR, and important somatic mutations found in CRPC are shown by arrows. The cell lines in which the mutations were identified are also indicated. Exons are shown as boxes. AR phosphorylation sites are marked with pinheads. Sumoylation sites are ahown as SUMO. Acetylation sites are marked as Ac. Tri-nucleotide repeat sequences are also shown. CAG-repeat, coding the poly Q tract, and GGN-repeat, coding the poly G tract, are indicated.

UTR, untranslated regions; TAU, transactivation unit; ARE, androgen response element. NTD, N-terminal domain; DBD, DNA-binding domain; NLS, nuclear localization signal. LBD, ligand-binding domain; AF, activation function.

Adapted with permission from: Waltering, K.K., A. Urbanucci, and T. Visakorpi, *Androgen receptor (AR) aberrations in castration-resistant prostate cancer*, in *Mol Cell Endocrinol*. 2012. Copyright (2012) Elsevier Ltd.

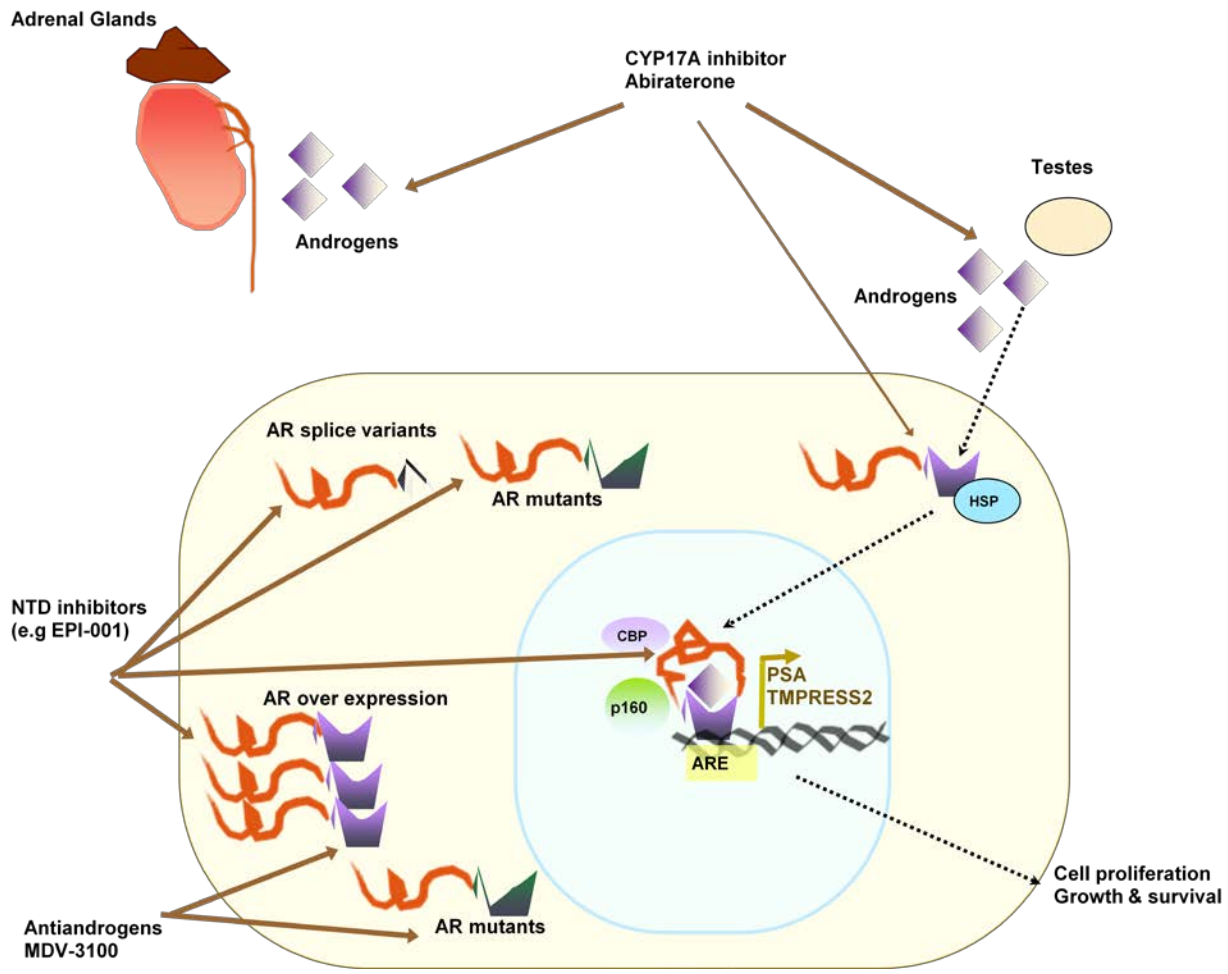

Figure 2. Mechanisms of resistance in CRPC and current therapies targeting the AR and androgen synthesis.

The main sources of androgen production are testicular and adrenal glands (androgens shown as diamonds). Androgens bind to the AR in the cytoplasm and result in conformational changes of the AR and dissociation from the heat shock proteins. AR then translocates to the nucleus, binds the androgen response element (ARE) of androgen regulated genes, interacts with coregulatory proteins, and regulates gene expression important for growth and survival of prostate cells. Mechanisms of resistance in CRPC include: AR amplification and/or over expression, AR mutations, expression of constitutively active AR splice variants lacking the LBD, and *de Novo* androgen synthesis.

Current therapies targeting the AR or androgen synthesis include: antiandrogens targeting the AR LBD (e.g. MDV3100), CYP17A inhibitors that block androgen synthesis (e.g. abiraterone acetate), and novel AR NTD antagonists (e.g. EPI-001 and SINT1). The intrinsically disordered AR NTD is shown in red. Arrows with solid lines indicate possible targets of drugs, and the proposed mechanisms of drug activity. Arrows with dotted lines outline the classical AR signalling pathway. NTD, N-terminal domain; LBD, ligand binding domain; HSP, heat shock protein.

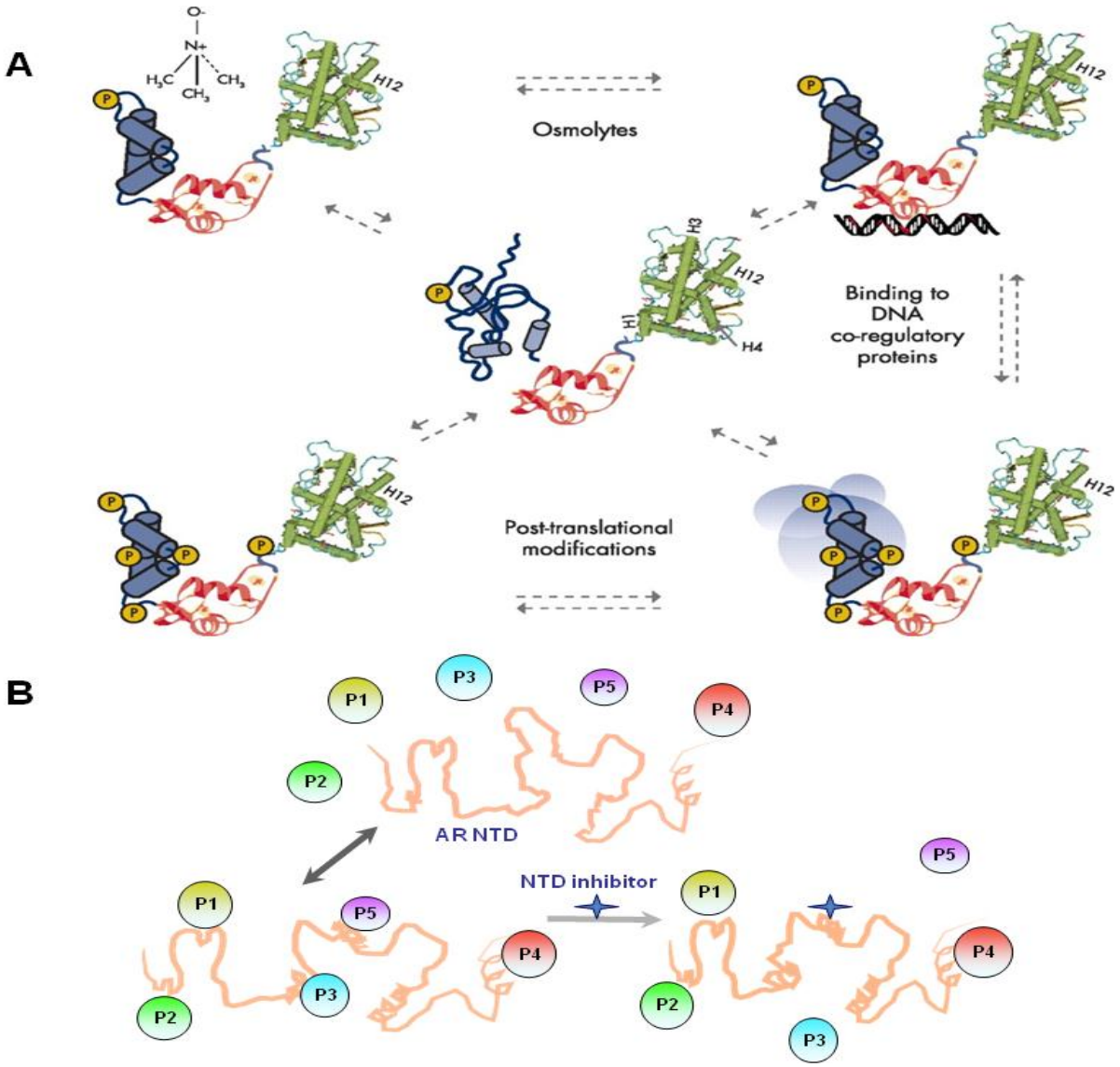

Figure 3. Folding of the SHR NTD and the proposed mechanism of the NTD inhibitors.

A) The steroid hormone receptor (SHR) NTD exists as an ensemble of conformations, having more or less stable structure (middle molecule). A more stably folded conformation of the NTD can be induced or selected by small molecules (osmolytes), posttranslational modification, DNA binding, and interactions with coregulatory proteins. In these models of NTD folding, the more stable structure is shown to be α-helical by the solid cylinders (blue). Reprinted with permission from: Kumar, R. and I.J. McEwan, *Allosteric modulators of steroid hormone receptors: structural dynamics and gene regulation.* Endocr Rev, 2012. **33**(2): p. 271-99. Copyright (2012) Endocrine Society. B) AR NTD interacts with multiple partner proteins (P1-5). A proposed mechanism of activity of the NTD inhibitors, EPI-001 or SINT1, is disruption of protein-protein interactions that are critical for activity of this intrinsically disordered region.

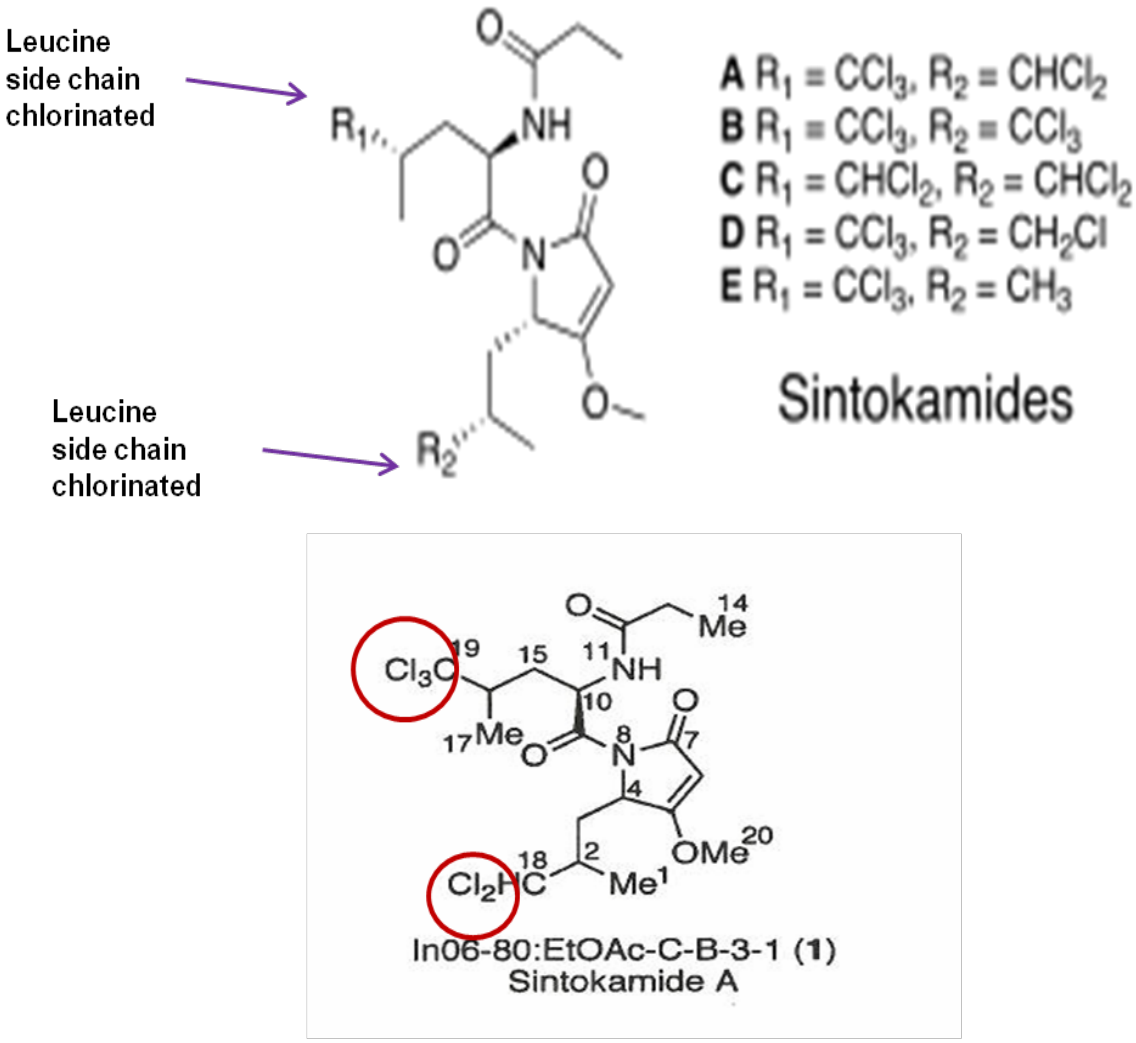

Figure 4. Structure of sintokamides.

The chlorinated peptides sintokamides A to E have been isolated from the marine sponge *Dysidea sp.* collected in Indonesia. Sintokamide A (SINT1) is an inhibitor of AR NTD in prostate cancer cells and is further studied in this thesis. Arrows indicated the leucine side chains. SINT1 has a total of five chlorines (indicated by red circle). Adapted with permission from Sadar, M.D., et al., *Sintokamides A to E, chlorinated peptides from the sponge Dysidea sp. that inhibit transactivation of the N-terminus of the androgen receptor in prostate cancer cells.* Org Lett, 2008. **10**(21): p. 4947-50. Copyright (2008) American Chemical Society.

Chapter 2 – Inhibition of Castration Resistant Prostate Cancer by Sintokamide A: an Antagonist of the Amino-Terminus of the Androgen Receptor

2.1 Introduction

Prostate cancer initially presents as a disease dependent on androgens and AR signaling, (with the exception of the neuroendocrine phenotype, which is negative for both fl-AR and variants). Therefore, ADT to prevent the production of androgens and inhibit the activation of AR is used for treatment of prostate cancer [193]. The duration of response to ADT, however, is brief and rising serum levels of PSA, expression of other AR target genes, and rapid growth of metastases commonly follows. This evidence suggests that AR is re-activated despite treatment with targeted therapies such as antiandrogen bicalutamide [97, 262-265]. The transition of the disease from androgen-dependent to CRPC has been studied for many years, and various mechanisms have been advanced to explain AR re-activation in CRPC. These mechanisms include amplification of AR gene and/or overexpression of AR protein [142, 145, 149, 151], gain of function mutations that result in a promiscuous AR that could be activated by antiandrogens [169, 170, 266], and alterations in AR co-activators and co-repressors.

Intra-tumoral synthesis of androgens and presence of adrenal androgens in the tumors have also been reported [25, 194, 196, 203, 267]. Based on this observation, new inhibitors of androgen production, targeting the CYP17 enzyme, such as abiraterone acetate, have been clinically approved [219]. These therapeutics demonstrated an overall survival increase in patients with metastatic CRPC [214].

Intriguingly, emergence of truncated AR variant proteins, speculated to be expressed via alternative splicing and/or DNA rearrangement mechanisms, is an additional mechanism of CRPC [181-185]. A number of these variants display constitutive, ligand-independent transcriptional activity; hence, their role in progression of CRPC is now under intense investigation. Clinically, increased mRNA expression of alternatively spliced AR variants in CRPC bone metastases is associated with shorter survival [268]. Additionally, tumors post abirateone treatment have higher levels of AR variant expression [269], emphasizing the expression of constitutively active AR splice variants as an important resistant mechanism in CRPC. Currently, no drugs for treating AR variant driven tumors exist. The majority of the known variants to date have exon 1 that encodes the NTD, but lack segments or the whole of LBD. Based on this observation, it is speculated that inhibitors of the AR NTD would be the first group of compounds that could potentially inhibit the activity of AR splice variants. Hence, development of inhibitors for this intrinsically disordered (ID) region of the AR is essential for effective therapies to inhibit AR and/or variant activity in CRPC. Consistent with these expectations, EPI-001, a potent small molecule inhibitor of AR NTD, is effective in blocking CRPC tumor growth *in vitro* and *in vivo*. Analogues of EPI-001 have also been successfully used for treatment of AR^{v567es} driven LuCaP tumor models and ARV7 driven VCaP xenografts (Sadar et al unpublished data). These promising results reconfirm the validity of NTD as a drug target and call for characterization and validation of other AR NTD inhibitors. SINT1, an unrelated class of molecules discovered by high throughput screening of marine sponge extracts is also a novel AR NTD inhibitor.

In this thesis, we provide multiple lines of evidence to show that SINT1 inhibits the activity of AR. We assess the effect of SINT1 on transcriptional activity of AR NTD and full-

length AR, proliferation and caspase 3/7 activities in LNCaP cells, and endogenous expression of PSA. Through studying the AR N/C interaction, AR nuclear translocation, and AR LBD binding assay, we provide evidence elucidating the mechanism of SINT1 inhibition. We also report the pharmacokinetics parameters of SINT1 and confirm its *in vivo* efficacy through blocking the growth of CRPC xenografts. Our results present a novel inhibitor of AR NTD that could be potentially developed as a therapeutic agent for the treatment of CRPC.

2.2 Materials and methods

2.2.1 Cell culture

LNCaP cells were provided by Dr. L.W.K. Chung (Cedars-Sinai Medical Center, Los Angeles, CA, USA). PC3 human prostate cancer cells and CV-1 monkey kidney cells were obtained from the American Type Culture Collection (Bethesda, MD). LNCaP cells were maintained in phenol red negative Roswell Park Memorial Institute medium (RPMI) with 5% fetal bovine serum (FBS), 100 units/mL penicillin and 100 units/mL streptomycin (antibiotics; Invitrogen, Burlington, ON, Canada). PC3 cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 5% FBS and antibiotics. CV-1 cells were maintained in phenol red negative Minimum Essential Media (MEM) media with 10% FBS, antibiotics, and 5% L-Glutamine. All cells were maintained at 37°C with 5% CO2.

2.2.2 Plasmids

PSA(6.1kb)-luciferase, PB-luciferase, Gal4DBD, p5Gal4UAS-TATA luciferase, and AR1–558Gal4DBD plasmids have been described [126, 135, 270]. ARR3-luciferase reporter was

prepared using the ARR3 fragment containing three androgen response regions, -244 to -96 region of the rat probasin gene.

PRE-E1b-Luc reporters and pCR3.1-hPR were gifts (C.L. Smith and N.L. Weigel, Baylor College of Medicine, Houston, TX); full-length GR expression plasmid (GeneCopoeia, Germantown, MD), pGRE-Luc reporter construct (Panomics, Fremont, CA), Gal4-ARLBD and VP16-AR-TAD constructs were gifts of Dr. K. Knudsen. To create the expression vector for AR-yellow fluorescent protein (YFP), full-length AR was cloned into the pEYFP N1 plasmid (Clontech). The clones were sequenced and expression of the fusion protein measured by western blot analysis.

2.2.3 Transfections and luciferase assay

LNCaP cells with passage numbers from 39 to 45 were seeded in Falcon (Becton Dickinson Laboratories, Franklin Lakes, NJ) 6, 12, or 24 well culture plates. Cells were incubated in RPMI 1640 with 5% FBS for 24 hours, resulting in 60-70% confluence. Plasmid DNA was transfected into cells using Lipofectin agent (Invitrogen) in serum-free media. Treatment was added 48 hours following transfection. Cells were pre-treated for 1 hr with bicalutamide (10 μM), SINT1 (5 μg/ml- 10 μM), or EPI-001 (10 μg/ml) before incubation with either synthetic androgen 1 nM R1881 (NEN Life Science Products Inc. Boston, MA) or ethanol carrier alone (0.000385% v/v), 50 μM FSK (Sigma-Aldrich Canada Ltd. Oakville, ON) or DMSO carrier alone (0.01% v/v) for an additional 24 or 48 hours.

Cells were harvested and lysed using passive lysis buffer (Promega, Madison, WI). Luciferase activities were measured using a commercial kit from Promega according to the manufacturer's protocol and activities were normalized to protein levels and expressed as relative

luminescent units/mg of protein/min. Transfection experiments were performed in 3 separate experiments using triplicate wells.

2.2.4 Transactivation of AR NTD

Transactivation of the AR NTD was measured in LNCaP cells co-transfected with the 5×Gal4UAS-TATA-luciferase and AR-(1-558)-Gal4DBD for 24 hr prior to pre-treatment with bicalutamide (10 μM), SINT1 (5 μg/ml- 10 μM), or EPI-001 (10 μg/ml) for 1 hr before incubation with FSK (50 μM) or vehicle for an additional 24 hr. Luciferase assay was performed as previously described.

2.2.5 Cell proliferation and *in vitro* caspase-3/7 activity

For the proliferation assay, LNCaP and PC3 cells were seeded in 96-well plates for 24 hr before pretreating for 1 hr with EPI-001 (10 μg/ml), bicalutamide (10 μM), or SINT1 (5 μg/ml-10 uM) prior to addition of 0.1 nM R1881 for LNCaP cells. BrdU incorporation was measured after 3 days for LNCaP and PC3 cells using BrdU ELISA kit (Roche Diagnostics).

For caspase-3/7 assay, LNCaP cells were seeded in 96-well plates and serum starved for 48 hr before pretreating for 1 hr with MDV3100 (0.4-2.4 μM) or SINT1 (4 -24 μM) prior to addition of 1 nM R1881 for an additional 48 hr incubation. Fluorescence intensity was measured using Apo-ONE® Homogeneous caspase-3/7 Assay (Promega) according to the manufacturer's protocol and was read using the Infinite M1000 (Tecan) with excitation at 499 nm and emission at 521 nm.

2.2.6 Fluorescence polarization

The Polar Screen Androgen Receptor Competitor Assay kit (Invitrogen) was employed according to the manufacturer's protocol with 20 nM AR-LBD and 2nM Fluoromone. The reactions were done in 40 µl aliquots in triplicates in Greiner 384 black clear bottom plates and fluorescence polarization read using the Infinite M1000 (Tecan) with excitation at 530 nm and emission at 590 nm.

2.2.7 Fluorescent microscopy

LNCaP cells were transiently transfected with an expression vector for AR-YFP for 24 hr using serum-free and phenol red-free RPMI media. Transfected cells were treated with DMSO, EPI-002 (10 µg/ml), bicalutamide (10 µM), SINT1 (5 µg/ml- 10 µM), or R1881 (1 nM) for 0, 30, 60 and 120 min. Cells were fixed with 4% paraformaldehyde at various time points, and mounted onto FisherBrand microscope slides with VECTASHIELD® fluorescence mounting medium with DAPI. The slides were examined by using a Zeiss Axioplan-2 Fluorescence Microscope (Zeiss, Toronto, ON, Canada).

2.2.8 EPI-002 and SINT1 combination

LNCaP cells with passage numbers from 39 to 45 were seeded in Falcon (Becton Dickinson Laboratories, Franklin Lakes, NJ) 24 well culture plates. Cells were incubated in RPMI 1640 with 5% FBS for 24 hours, resulting in 60-70% confluency. Total of 18 µg plasmid DNA per plate was transfected using Lipofectin agent (Invitrogen) in serum-free media. Treatment was added 48 hours following transfection as follows. Cells were pre treated for 1hour with SINT1 (0-24 µM), or EPI-002 (0-35 µM) or SINT1 and EPI-002 combination with a

constant ratio of 1.3 for EPI-002/SINT1 concentration. Cells were then incubation with synthetic androgen 1 nM R1881 (NEN Life Science Products Inc. Boston, MA) for 48 hr and luciferase assay was performed as described.

2.2.9 Endogenous expression of androgen-regulated genes

LNCaP prostate cancer cells were grown under serum-free and phenol red-free conditions for 48 hr and pretreated for 1 hr with vehicle, bicalutamide (10 μM), SINT1 (5 μg/ml- 10 μM), or EPI-001 (10 μg/ml) before addition of 1 nM R1881 for 16 hr. Total RNA was extracted using Qiagen RNeasy Mini Kit and cDNA was synthesized by Invitrogen Reverse Transcription assay (Invitrogen Life Technologies, Carlsbad, CA, USA). Quantitative real-time (qRT)-PCR was performed separately in triplicates for three biological samples. Levels of expression PSA transcript was measured and normalized to the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) housekeeping gene. Real-time amplification was performed with initial denaturation at 95°C for 2 min, followed by 40 cycles of two-step amplification (95°C for 15 sec, 55°C for 30 sec). Primer sequences for real-time PCRs were as follows: GAPDH: 5-CTGACTTCAACAGCGACACC-3 (forward) and 5- TGCTGTAGCCAAATTCGTTG-3 (reverse). PSA: 5-CCAAGTTCATGCTGTGTGCT-3 (forward) and PSA 5-CCCATGACGTGATACCTTGA-3 (reverse).

2.2.10 Steroid receptor specificity

Transcriptional activities of endogenous human AR were determined in transient transfection assays using the PSA(6.1kb)-luciferase reporter. PR and GR transcriptional activities were measured in LNCaP cells that were transfected with expression vectors for PRβ and GRα with respective PRE-luc or GRE-luc reporter gene constructs in phenol red-free media. Cells

were pre-treated with bicalutamide (10 μM), SINT1 (5 μg/ml- 10 μM), or EPI-001 (10 μg/ml) for 1 hr before incubation with vehicle, (10 nM) dexamethasone (GR), or (10 nM) 4-pregnene-3,20 dione (progesterone) (PR), and (1 nM) R1881 (AR) for an additional 24 h.

2.2.11 AR N/C interaction

CV-1 cells ($2.5x10^4$ cells/well in 24-well plates) were grown in MEM media with 10% FBS as described above. Two-hybrid N/C interaction assay was used. Cells were transfected using Fugene6 reagent (Roche Diagnostics, Laval, Quebec, Canada) with 0.25 μg/well 5XGAL4Luc reporter vector, VP-AR-(1–565) (0.25 μg/well) that encodes the VP16 transactivation domain fused to amino residues 1-565 of AR NTD, and GAL4DBD-AR628–919 (0.25 μg/well) encoding the wild-type AR LBD C-terminus amino acid residues 628-919 fused to the Gal4 DBD. Transfected cells were incubated for 24 hr with 1 nM R1881 or vehicles after 1 hr pre-treatment with bicalutamide (10 μM), SINT1 (5 μg/ml- 10 μM), or vehicle control.

2.2.12 Animals

Male non-obese diabetic-severe combined immunodeficient (NOD-SCID) mice, 6-8 weeks of age, were obtained from the breeding program at the Animal Research Centre of the British Columbia Cancer Agency. All experiments involving animals conform to the relevant regulatory standards and the experiments were approved by the University of British Columbia Animal Care Committee (Vancouver, BC, Canada).

2.2.13 Pharmacokinetics study

Pharmacokinetics assessment of SINT1 was done at NAEJA Pharmaceutical Inc. A total of 27 female CD-1 mice received a single intravenous SINT1 dose of 50 mg/kg. The animals were

evaluated for changes in clinical signs. Blood samples were collected at 2.5min, 5min, 15 min, 30 min, 45 min, 1hr, 2hr, 4hr, & 8hr post dosing by randomizing 3 mice per time point. Single dose sample was prepared using tolbutamide as internal standard for utilizing an LC/MS/MS method developed at NAEJA Pharmaceutical Inc. Plasma concentration of SINT1 for each time point was reported.

2.2.14 Xenografts

Male NOD-SCID mice, 6-8 weeks of age, were subcutaneously inoculated with LNCaP cells suspended in 75 µl of RPMI 1640 (5% FBS) with 75 µl of Matrigel (Becton Dickinson Laboratories). When tumor volume averaged approximately 100 mm^3, castration was performed by making a small incision in the scrotum to remove each testicle following ligation of the cord. Seven days after castration, animals were injected intratumorally (i.t.) with 30mg/kg body weight of SINT1 every 3 days (DMSO group were treated every 5 days) for a total of 15 days. Volume of compound or control was about 50 µl per tumor. Tumor volumes were calculated by the formula length × width × height × 0.5236. Tumors and major organs were excised 5 days after the last intratumoral (i.t.) injection and prepared for immunohistochemistry. Animal behavior and body weight were monitored throughout the study period for signs of toxicity.

2.2.15 Immunohistochemistry

Xenografts were fixed in 10% neutral buffered formalin, processed through alcohols, and embedded in paraffin. For immunohistochemical staining, tissue sections (5 µm) were blocked in immunohistochemistry solution (Immunovision Technologies, Brisbane, CA) and staining was carried out by *Wax it Histology Inc*. using anti–Ki-67 antibody and hematoxylin and eosin (H&E) staining.

2.2.16 Statistical analysis

To identify significant changes in response to various treatments, we used the Two-sample Student's T-test. All calculations were done using GraphPad Prism software. A p-value cut-off of 0.05 was employed for all tests.

2.3 Results

2.3.1 SINT1 reduces proliferation of LNCaP cells but not PC3 cells

In a previous report, we demonstrated that SINT1 blocked the androgen dependent proliferation of LNCaP cells while having no effect on PC3 cells [123]. Here we confirmed this observation by including EPI-001 as a positive control. Effect of SINT1 on androgen dependent proliferation of LNCaP cells was similar to that of EPI-001, a first-in-class inhibitor of AR NTD, and bicalutamide, an antiandrogen commonly used for treatment of prostate cancer patients. We also examined the effect of SINT1 in blocking proliferation in PC3 human prostate cancer cells that do not express functional AR and do not rely on the AR for growth and survival. SINT1 showed no effect on proliferation of PC3 cells similar to EPI-001. This indicates that the effects of SINT1 on proliferation are specific to prostate cancer cells that express AR. These data support the specificity of SINT1 for targeting the AR which resulted in blocking AR-dependent proliferation of cancer cells (Figure 5A and 5B).

2.3.2 SINT1 blocks forskolin-induced transactivation of the AR NTD

AR NTD is essential for transcriptional activity of the AR. Jenster and colleagues demonstrated that this domain is critical for both ligand dependent (in the presence of androgens) and ligand independent activation of AR [40]. In the absence of androgens, AR NTD can be

activated by forskolin (FSK) [135], which stimulates PKA activity. To test the ability of SINT1 for inhibiting the transactivation of the AR NTD, LNCaP cells were transfected with an expression vector of human AR NTD encoding amino acids 1–558 fused to the Gal4DBD and a reporter construct with a Gal4-binding site. The inhibitory effect of EPI-001 on this chimera has been reported [122]; hence this compound was employed as a positive control. SINT1 reduced FSK-induced transactivation of the AR NTD to baseline levels similar to that of EPI-001. Consistent with a previous report, SINT1 is a first-in-class and structurally unrelated compound to EPI-001 that blocks FSK-induced transactivation of the AR NTD in LNCaP cells (Figure 5C) [123].

2.3.3 SINT1 increases apoptosis in LNCaP cells by activation of caspase-3/7

In vitro analysis of apoptosis employed a caspase-3/7 activity assay. This assay measures the intensity of a fluorescent end product that is produced following cleavage of a profluorescent substrate by the caspase-3/7 enzyme. The cysteine aspartic acid-specific protease (caspase) family of proteins play key roles in apoptosis in mammalian cells [271-273]. Active caspases are involved in cleavage of essential biological substrates including poly-(ADP ribose) polymerase (PARP), DNA-dependent protein kinase (DNA-PK), topoisomerases, and protein kinase C (PKC) [274, 275]. Once these key enzymes are cleaved, essential cellular mechanisms such as DNA repair would be disabled which eventually causes cell death. To test the effect of SINT1 on the activity of caspase-3/7 enzymes, LNCaP prostate cancer cells were treated with SINT1 (4-24 µM) or MDV-3100 (0.4-2.4 µM) for 1 hr followed by 48 hr of treatment with 1 nM R1881. We observed a 2-fold increase in activity of caspase-3/7 in SINT1 treated samples compared to the control treatment at 24 µM (Figure 5D). Consistent with a previous report that MDV3100 induces apoptosis [276], here MDV3100 also showed a dose dependent increase in apoptosis

with the highest increases at 1.8 and 2.4 μM. However, as MDV3100 is used at very low concentrations (0.4-2.4 μM) compared to the commonly used 10 μM, these results did not reach statistical significance.

2.3.4 SINT1 suppresses endogenous expression of androgen regulated genes

Thousands of potential AR regulated genes have been mapped that emphasize the crucial role of the receptor in regulating many biological processes [277, 278]. To test whether the inhibitory effect of SINT1 on the AR NTD had any effect on the endogenous gene expression, we employed a qRT-PCT assay. We looked at the levels of PSA transcript, a well-characterized androgen-regulated gene in LNCaP prostate cancer cells. Cells were treated with SINT1, EPI-001 or bicalutamide for 1 hr and then treated with R1881 for 16 hr to induce androgen-regulated gene expression. Interestingly, androgen induced expression of this gene was blocked when treated with SINT1 to levels similar to that obtained with both bicalutamide and EPI-001 (Figure 6A).

2.3.5 SINT1 specifically inhibits the AR and not other steroid receptors

To determine if SINT1 would inhibit GR or PR transcriptional activities, LNCaP prostate cancer cells were cotransfected with expression plasmids for full-length human GR, PRβ, and the relevant gene reporter constructs. Cells were treated with ethanol vehicle, dexamethasone (GR), or 4-pregnene-3, 20 dione (progesterone) (PR), and R1881 (AR) to transactivate each receptor. SINT1 did not inhibit either PRE-luciferase or GRE-luciferase activities (Figure 6B), whereas bicalutamide inhibited PR as expected due to the homology of the LBDs of the receptors. Consistent with our previous observations, SINT1 significantly decreased the activity of AR as

measured by PSA (6.1)-Luc reporter. This data emphasizes that inhibitory effect of SINT1 is specific to the AR, and does not inhibit other steroid receptors.

2.3.6 SINT1 inhibition of AR activity is additive to EPI-002

With the introduction of novel antagonists of AR that target the NTD, it is likely that combination therapy of NTD could result in the best therapeutic effect by inhibiting the AR at multiple regions. As with c-myc inhibitors [241], we believe that different chemical classes of AR NTD inhibitors may work on distinct regions of the NTD thereby eliciting a different molecular mechanism of inhibition. Although the direct binding sites of these inhibitors are yet to be determined, we hypothesized that EPI and SINT1 compounds have different mechanisms of inhibition as these are structurally unrelated chemical classes. Hence we tested the relative efficacy of EPI-002 and SINT1 compounds in combination studies. The PSA-luciferase, probasin-luciferase and ARR3-luciferase reporter constructs were transfected in LNCaP cells. Pre-treatment was performed with SINT1 at 3-24 uM range, EPI-002 at 4-35 uM range, and combined at 1.3:1 molar ratio of EPI-002:SINT1 for 1 hr followed by 48 hr R1881 treatment. Both SINT1 and EPI-002 blocked the androgen induction of all 3 luciferase reporters. Importantly, the combination of the two compounds showed an additive effect on all 3 reporters at the four highest concentrations excluding the highest concentrations (Figure 7A). Additive effect was determined based on the assumption of first order binding kinetics of AR NTD and SINT1 or EPI-002 (m=1) [279]. The morphology of LNCaP cells showed no obvious signs of toxicity (Figure 7B).

The IC_{50} of SINT1 for inhibition of AR transcriptional activity was determined to be 10.74 ± 2.44 μM on PSA 6.1-Luc reporter, 11.70 ± 1.82 μM on PB-Luc reporter, and 13.32 ± 0.93 μM on ARR3-Luc reporter (Table 1).

2.3.7 SINT1 does not bind the LBD of AR

To determine whether SINT1 affects ligand-binding, we employed a fluorescence polarization assay that is based on competition of inhibitors for the LBD with a fluormone that binds the recombinant AR-LBD. Bicalutamide, MDV3100, and the synthetic androgen R1881 are all known to interact with the LBD and bound to the AR-LBD as expected (Figure 8A). However, EPI-002, a first in class inhibitor of the AR NTD did not compete with the fluormone for binding of the AR-LBD in the concentration range of 0.5 nM to 50 μM consistent with previous reports [122]. Importantly, SINT1 in the same concentration range of 0.5 nM to 50 μM showed a similar trend to EPI-002. These data suggest that SINT1 does not interact with the AR-LBD and does not show any effect on ligand binding.

2.3.8 SINT1 blocks AR N/C interaction

Transcriptional activity of the AR involves multiple steps and cofactors. Interaction between the N and C termini of the receptor in response to ligand is a crucial step that stabilizes the receptor and facilitates the conformational changes required for protein-protein interactions. Previous studies have shown that anti-androgens like bicalutamide disrupt N/C interaction induced by R1881 [280]. To assess whether SINT1 affects androgen-induced N/C interaction, we employed a mammalian two-hybrid system in AR null CV1 cells. An expression vector for a chimera protein of amino acids 1–565 of the AR NTD fused to VP16 (N terminus), an expression vector for the Gal4DBD fused to the amino acids 628–919 of the AR (C terminus)

and the Gal4-luciferase reporter were cotransfected in the cells. Cells were pre-treated with vehicle control, bicalutamide, or SINT1 for 1 hr followed by induction with R1881 for 24 hr. As expected, R1881 stimulated the N/C interaction as measured by increased luciferase activity. Antiandrogen bicalutamide blocked this activity and SINT1 inhibited this interaction by ~ 40% (Figure 8B).

2.3.9 AR nuclear translocation is not induced by SINT1

Upon binding of ligand, AR is dissociated from heat shock proteins in the cytoplasm and is translocated to the nucleus by interaction with the importin protein. Nuclear AR binds androgen response elements (AREs) in the enhancer or regulatory regions of androgen regulated target genes and interacts with co-regulator proteins required for its transcriptional activity [44]. To assess the effect of SINT1 on AR nuclear translocation, we transfected LNCaP cells with an expression vector for AR tagged with yellow fluorescent protein (AR-YFP). Cells were treated with vehicle control, bicalutamide, EPI-002, SINT1, or R1881. In vehicle-treated cells, AR-YFP was predominantly localized to the cytoplasm. The receptor was mostly localized in the nucleus after treatment with R1881. Bicalutamide treatment caused the AR-YFP to be localized in the nucleus. Importantly, SINT1 and EPI-002 did not cause translocation of the receptor to the nucleus and AR-YFP remained predominantly cytoplasmic upon treatment with these inhibitors of the NTD (Figure 8C).

2.3.10 Pharmacokinetic evaluation of SINT1 *in vivo*

The peptide bond in the structure of SINT1 would allow this compound to be readily metabolized in the body. Rapid metabolism would be an indication of the need for chemical modification to increase stability. We evaluated the pharmacokinetics of this compound for

future *in vivo* studies. SINT1 was administered to mice at an i.v dose of 50 mg/kg (n=3 per time point) at 9 time points. The concentration of the drug in the plasma was measured at 0.04, 0.08, 0.25, 0.50, 0.75, 1, 2, 4, and 8 hr. Maximum concentration of the drug in plasma, C_{max}, was 4.1 µg/ml (~ 8 uM) which is slightly lower than the IC_{50} of the compound *in vitro*. The half-life of the drug was determined to be 1.2 hr (Table 2 and Figure 9).

2.3.11 SINT1 causes regression of CRPC tumors *in vivo*

In vivo experiments were done to test whether SINT1 would have an effect on CRPC tumor growth. Male mice bearing LNCaP prostate cancer subcutaneous xenografts were castrated when tumors were 100 mm^3 (mean=123.3± 27.4 mm^3; n=18) and randomized into two groups. One week after castration, the animals were treated every 3 days with an intra-tumoral dose of SINT1 (30 mg/kg) or matching volume of vehicle dimethyl sulfoxide (DMSO) every 5 days for a total of 15 days. We selected the intra-tumoral delivery to obtain the highest concentration of SINT1 in the tumor based on the pharmacokinetics data and due to rapid metabolism of the compound. Importantly, in spite of the current labile structure, we observed that 3 out of 7 SINT1 treated CRPC xenografts regressed and 2 other animals showed cytostatic effects which were most pronounced between 9 and 12 days (mean = 103.3± 11.97 mm^3, n=7) compared to DMSO-treated tumors (n=11) that grew to 153.7± 11.87 mm^3 (p=0.011) (Figure 10A and 10B). The SINT1 treated tumors had a less bloody appearance (Figure 10C). No change in animal body weight was detected upon the duration of the experiment (start: 24.6±1.1 grams; finish: 25.0±1.4 grams) suggesting that SINT1 is not generally toxic to the animals (Figure 10D).

Immunohistochemistry (IHC) for proliferation marker Ki67 suggested that SINT1 reduced proliferation (Figure 10E). These data are consistent with the reduction of tumor volume in response to SINT1compared to control-treated tumors.

2.4 Discussion

Currently, there is no cure for advanced prostate cancer. All current therapies, with survival benefits of a few months, eventually fail and unfortunately, patients succumb to lethal CRPC [27, 281]. The continuous importance of AR has been reported in progression of CRPC. Despite the discovery of new therapeutics for CRPC in the last few years, the need for novel therapies targeting alternative molecular targets still exists. Functional AR NTD is necessary for AR transcriptional activity and is implicated in the underlying molecular mechanism of CRPC. Proof of concept studies with EPI-001 confirmed AR NTD as a unique therapeutic target for CRPC [122].

In this thesis, a novel class of inhibitors targeting the NTD of the AR is introduced and the activity and safety of this compound is demonstrated through proof of principle studies. The transactivation of AR NTD and full-length AR were reduced and androgen-dependent proliferation of LNCaP prostate cancer cells was inhibited in response to treatment with SINT1. SINT1 caused an increase in apoptosis by activation of caspase-3/7. Consistent with the hypothesis that this group of inhibitors function through an alternative mechanism than anti-androgens, SINT1 did not cause AR nuclear translocation and did not bind the AR LBD *in vitro*. Effect of SINT1 was additive to EPI-002, another class of NTD inhibitors, suggesting the possibility of existence of multiple hot spots in this IDP [237, 238]. PR and GR transcriptional activity were not affected in the presence of SINT1, indicating the specificity of this compound

for the AR. *In vivo*, SINT1 had a half-life of 1.16 hr and inhibited the growth of LNCaP xenografts.

Comparing the results of SINT1 studies with EPI-001 indicates that the two compounds have many similar biological effects, for example, both inhibit the N/C interaction, do not bind the LBD, and do not cause nuclear translocation of the AR [122]. This highlights the unique mechanism of NTD inhibitors as opposed to the antiandrogens, including bicalutamide and MDV3100, which function through binding the LBD and causing AR nuclear translocation. However, the additive effect of EPI-001 to SINT1 suggests that the two compounds are not in competition for a common binding site. This shows that multiple binding sites within the ID region of AR exist and can be targeted independently. Our results are consistent with the observations from the Metallo group in development of c-myc small molecule inhibitors. Three binding sites in ID regions of c-myc have been identified that can act as binding sites for 7 small molecules, independently and simultaneously [240, 241, 244].

The biological response to SINT1 can differ from antiandrogens, bicalutamide and MDV3100, which results in the following advantages for treatment with this compound. SINT1 has a shorter half-life of 1.16 hr in mice when compared to other AR antagonists. EPI-001 has a half-life of 3.4 hr in mice which is longer than SINT1, but shorter than antiandrogens. The half-lives of SINT1 and EPI-001 are yet to be determined in humans (in clinical trials), and profound differences between human and mice in metabolism, serum protein-binding, and pharmacokinets could result in longer half-lives of SINT1 and EPI-001 in humans. However, relatively shorter half-lives compared to antiandrogens are expected. Serum half-life of MDV3100 is approximately 1 week (3 – 13 days in individual patients), and a structurally similar compound to MDV3100, RD162, is reported to have a serum half-life of about 30 hr in mice [57].

Bicalutamide's half-life is about 6 days [282], and abiraterone acetate's terminal half-life is in the range of 5 to 14 hrs in patients [283]. In general, lower accumulation and toxicity of compounds is associated with shorter half-life. Hence both SINT1 and EPI-001 would be expected to have a different toxicity profile compared to other compounds. Historically, side effects such as [284] hepatotoxicity and [224, 285] gynecomastia [223, 286] have been reported in subset of patients treated with bicalutamide, and concerns about seizures are raised regarding MDV3100 treatment [287]. The unique mechanism of action of NTD inhibitors suggests that common side effects of antiandrogens would not be expected following treatment with these compounds; however, a comprehensive toxicity profile must be determined in other pre-clinical animal studies and clinical trials.

Additionally, specificity of the NTD inhibitors for the AR appears to be advantageous to the antiandrogens. Bicalutamide is known to inhibit the PR and the effect of MDV3100 on this receptor has not been reported [276]. Although, both PR and GR are known to share common interacting proteins such as CBP [288], specific inhibition of AR and not other steroid receptors is consistent with our data that SINT1 targets the NTD, as this region shares the least homology between nuclear hormone receptors. Lack of effect of SINT1 on the proliferation of AR negative PC3 cells, and lack of effect on body weight or behavior of animals receiving SINT1 also indicate specificity of this compound.

Another advantage of SINT1 could be based on the observation that recent studies have found that many cancer associated mutations are likely to occur within ordered regions of proteins [289]. Based on this observation, mutations in the IDPs including the AR NTD could be less likely following treatment with SINT1 or EPI-001 and/or such mutations are likely to not impair the function of the protein due to its flexible nature. AR gain of function mutations

following antiandrogen treatment is an important mechanism underlying CRPC. Such gain-of-function mutations result in a promiscuous receptor that is activated by using antiandrogens [47]. The H874Y mutation found in patients treated with flutamide and W741C mutation found in bicalutamide treated patients are well-studied cases of this resistance mechanism [67].

The AR NTD contains the AF-1 region that is necessary for AR transcriptional activity [34, 96]. Thus targeting the NTD would block AR regardless of ligand [122]. However, NTD inhibitors may have other effects on the LBD. Studies have shown that the function of LBD is independent of other domains when binding ligand [40]. However dissociation of the ligand and stability of the receptor is affected by the N/C interaction. N/C interaction causes slower dissociation of bound ligand [62] by changing the dissociation kinetics. Deletion of the NTD increases the dissociation rate of bound ligand [80]. Hence, even though EPI-001 and SINT1 do not directly interact with the LBD, they could affect the ligand dissociation by preventing N/C interaction.

Regression of CRPC by a single agent has historically been difficult to achieve. Most drugs for CRPC would have no effect when administered alone and combination with taxanes would be required for any therapeutic effect. Only recently, novel AR antagonists such as MDV3100 and EPI-001 have overcome this barrier and demonstrated excellent efficacy in animal studies with regression of CRPC xenografts. The apoptotic effect of EPI-001 and MDV3100 is consistent with effective blockade of AR activity. EPI-001 caused increased apoptosis in CRPC animal models shown by regression of tumors and significant increase in caspase-3 staining [122] of the tumors. Similar to EPI-001, in LNCaP xenograft model of CRPC, MDV3100 causes a dose-dependent decrease in tumor volume. MDV3100 induces cleavage of poly (ADP-ribose) polymerase (PARP) in prostate cancer cells. Bicalutamide has no effect of PARP cleavage and

shows a minimal effect with no tumor regression in animal studies [30, 233, 276]. MDV3100 has also shown significant survival benefit in clinical trials [30], consistent with the preclinical observations in animal models. Increase in apoptosis observed in our *in vitro* studies, in addition to the regression of tumor volume in the xenograft model indicate that a SINT1 analogue has the potential to be used as a therapeutic for effective clinical management of CRPC.

2.5 Ongoing and future studies

Following this promising data, next steps in further investigation of the mechanism of SINT1 activity and assessment of this compound as a clinical candidate could be as follows.

A comprehensive study of the underlying molecular mechanisms of activity of SINT1 is yet to be performed. Although essential steps for AR transcriptional activity such as AR nuclear translocation and N/C interaction have been shown to be inhibited by SINT1, the effect of this compound on other protein-protein interactions and AR DNA interactions is yet to be elucidated. To exploit the effect of SINT1 on AR interaction with AREs, *in vivo* chromatin immunoprecipitation (ChIP) assay combined with microarray analysis (ChIP-chip) could be used. Global mapping of alterations in AR/ARE interactions would provide a unique profile for SINT1 treatment, which will be a key factor in understanding the mechanism of inhibition of CRPC by SINT1. To investigate the effect of SINT1 on protein-protein interactions, co-immunoprecipitation mass spectrometry (CoIP MS) techniques could be employed. Similar to the ChIP-chip approach, a global profile of protein interactions blocked or induced by SINT1 treatment would be generated which further illustrates the mechanisms of activity of this compound. Such experiments would be expected to yield data showing alteration in protein interactions including common transcription factor and cofactors, as well as novel protein

interactions due to the ID nature of AR and possibility of multiple partner proteins [34]. Other data generated from a global CoIP MS approach would be elucidation of the effect of inhibitor binding on the post translational modifications (PTM) of the NTD. As ID proteins are known to have multiple PTM, inhibitors that effectively bind these regions are expected to alter the PTM resulting in protein stability and function variations [26, 94, 188].

A next step in mechanism studies would be to map the region of AR NTD that SINT1 binds and investigate the nature of this binding. Although the lack of a crystal structure of the NTD makes these experiments difficult to carry out, *in vitro* binding assays using Click chemistry techniques and expression vectors spanning smaller regions of NTD and AF1 are possible methods [18].

To further confirm the role of SINT1 in inhibition of CRPC, additional functional experiments are required. The most critical experiments would involve the AR splice variants that have been reported recently [139]. As the expression and constitutive activity of these splice variants have been confirmed as a resistance mechanism to current therapies such as abiraterone [269], and based on the evidence that SINT1 inhibits the transactivation of AR NTD in LNCaP cells, it is expected that SINT1 inhibits the transcriptional activity of AR splice variants as well. Experiments testing this hypothesis would involve cell lines expressing the constitutively active variants such as VCaP and LNCaP95, which express AR V7, and animal models such as LuCaP 86.2 that has been shown to solely express AR^{v567es} [182]. Demonstrating the effect of SINT1 on AR variants would add this drug to the category of AR antagonists that could potentially be used to treat patients that fail abiraterone therapy [122].

Finally, to exploit the possibility of developing SINT1 as a therapeutic option for clinical trials, further pre-clinical studies are required. With the promising results in the proof of concept

studies, an important next experiment would be to investigate the efficacy of SINT1 in long term *in vivo* studies, such as studies that yield Kaplan–Meier plots to show survival benefits of the compound compared to current treatment options. As the $t_{1/2}$ of the compound is relatively short, pilot studies would be required to determine the best delivery method. Longer treatment periods obtained by oral dosing or i.v. injections, could provide an indication if tumors will completely regress in response to SINT1 and if this treatment would be safe in long term. Similar experiments with tumors expressing AR variant such as VCaP xenografts or mixed population of AR full-length and variants, such as LuCaP tumors would also confirm the efficacy and potency of SINT1 for treatment of different patient populations [182, 269]. Effect of SINT1 on AR negative tumors, such as PC3 xenografts may also be studied to translate the results from specificity of SINT1 shown in cell lines to *in vivo* models.

All in all, data gathered from these proposed studies could establish SINT1 as a valid therapeutic candidate for CRPC treatment. Curiously, SINT1 and EPI-001 are first in class compounds with a distinct mechanism for targeting AR; hence effects of these compounds on patients in clinical trials would be of great interest. If the pre-clinical results of these compounds could be translated in the clinic, a novel and promising class of drugs is introduced which could provide the patients with an option following failure of current antiandrogens.

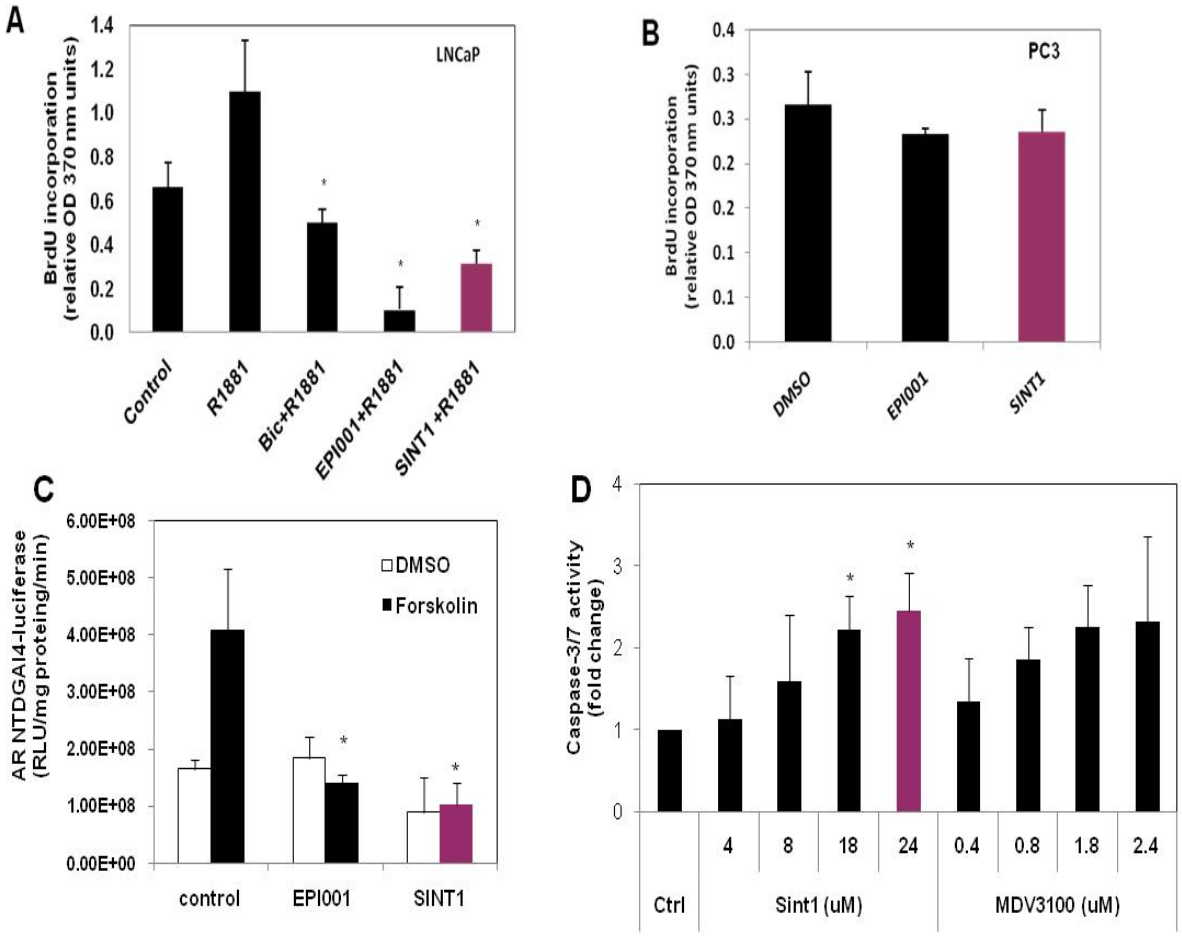

Figure 5. SINT1 inhibits AR-dependent proliferation, blocks transcriptional activity of AR NTD, and increases caspase-3/7 activity.

A and B) LNCaP cells treated with bicalutamide (Bic) (10 µM), EPI-001 (10 µg/ml) or SINT1 (10 µM) for 1 hr prior to 0.1 nM R1881 induction for 3 days. SINT1 does not block proliferation of PC3 cells treated with vehicle, EPI-001, or SINT1 for 3 days. Statistics compare each treatment to R1881 treatment (A) and DMSO treatment (B). The error bars represent the mean ±SD. Data representative of 3 experiments.

C) Transactivation assay of the AR NTD was performed in LNCaP cells cotransfected with p5 × Gal4UAS-TATA-luciferase and AR-(1-558)-Gal4 DBD, prior to incubation with 50 µM forskolin (FSK) for 24 hr. SINT1 (10 µM) or EPI-001 (10 µg/ml) were added 1h before the addition of FSK. Statistics compare each treatment to control. The error bars represent the mean ±SD. Data representative of 3 experiments.

D) SINT1 induces caspase-3/7 activity. LNCaP cells were pretreated with SINT1 or MDV3100 for 1 hr prior to 1 nM R1881 treatment for 48 hr. Concentrations are reported in µM. Statistics compare each treatment to control. The error bars represent the mean ± SEM. Data average of 3 experiments.

Student's t test: $^{*}p < 0.05$.

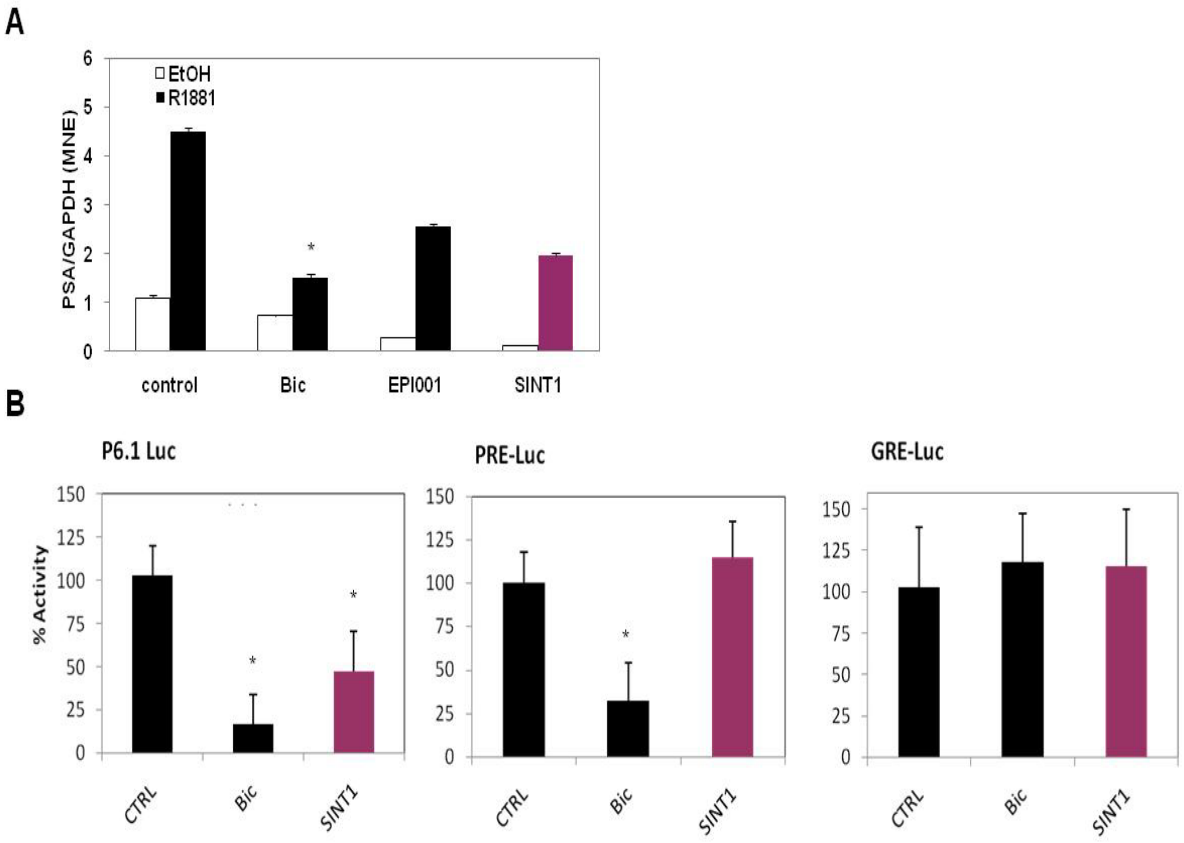

Figure 6. SINT1 inhibits endogenous expression of PSA and its effect is specific to AR but not other steroid hormone receptors.

A) LNCaP cells were pretreated with bicalutamide (Bic) (10 µM), SINT1 (10 µM), or EPI-001 (10 µg/ml) prior to incubation for 16 hr with 1 nM R1881 and harvesting total RNA. Levels of PSA mRNA were measured by qRT-PCR normalized to GAPDH mRNA. The error bars represent the mean ± SD. Data representative of 3 experiments.

B) Ligands were 1 nM R1881 for PSA 6.1 luciferase reporter, 10 nM PRE-luc 4-pregnene-3,20 dione (progesterone) for PRE-Luc assay and 10 nM dexamethasone for GRE-luc assay. Control is treated with ligand only. Y axis indicating % activity, calculated based on luciferase activity (RLU/mg protein/min) of treatment groups normalized to the control. The error bars represent the mean ± SEM. Data average of 3 experiments.

Student's t test: $^{*}p < 0.05$.

CTRL, control; PR, Progesterone Receptor; GR, Glucocorticoid Receptor; Luc, luciferase; PRE, progesterone response element; GRE, glucocorticoid response element.

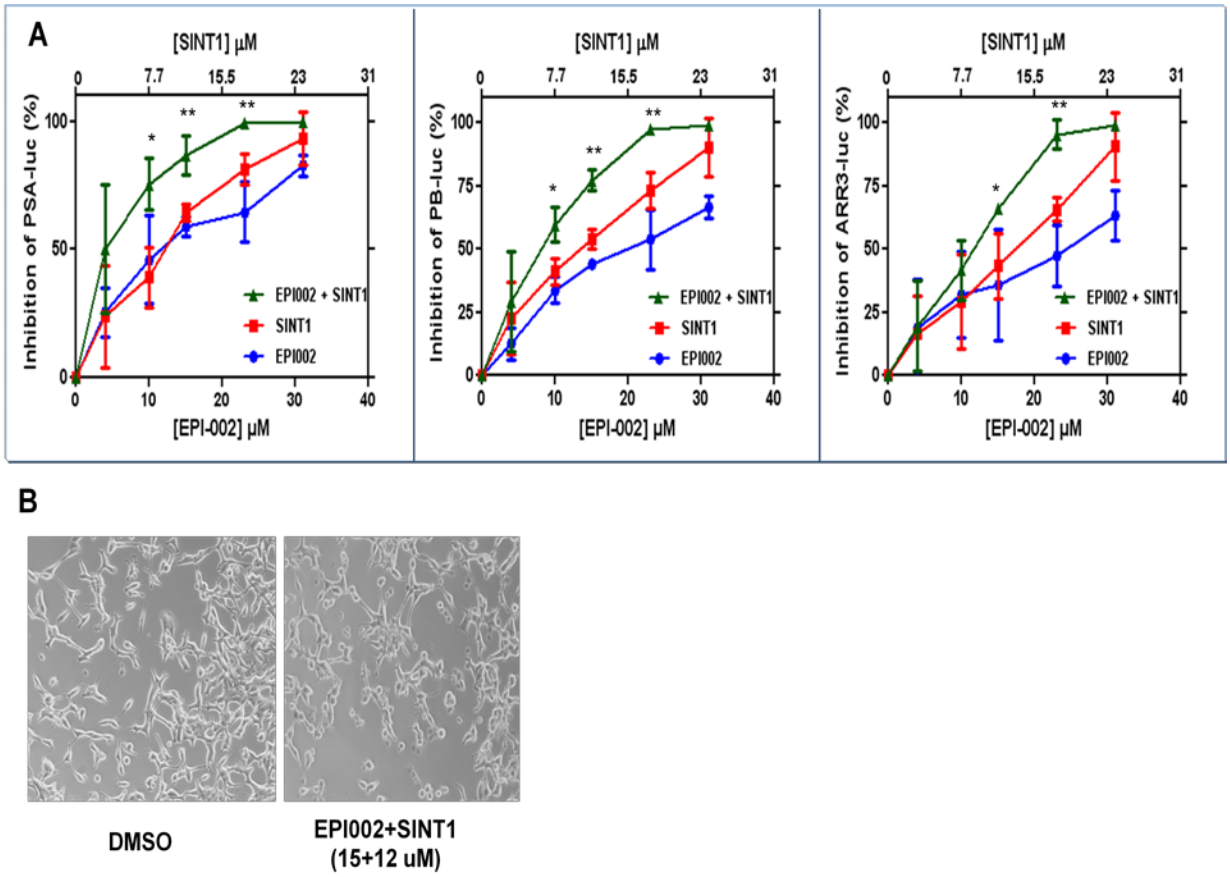

Figure 7. SINT1 inhibition of AR transcriptional activity is additive to EPI-002.

A) Effect of SINT1 (0-24uM) and EPI-002 (0-35uM) alone or in combination (1.3/1 ratio of EPI-002/SINT1) on androgen-induced AR transactivation in LNCaP cells transfected with the PSA(6.1kb)-luc, ARR3-luc, or PB-luc reporters. Pretreated with compounds for 1 hr followed by 1 nM R1881 treatment for 48 hr. Y axis indicating % inhibition, calculated based on luciferase activity (RLU/mg protein/min) of treatment groups normalized to the control. Statistics compare SINT1 to SINT1+EPI-002 treatment at each dose. The error bars represent the mean ± SEM. Data average of 3 experiments. Student's t test: $^{**}p < 0.01$ $^{*}p < 0.05$.

B) Images of LNCaP prostate cancer cells prior to harvest 48 hr after treatment as above.

Table 1. IC_{50} of SINT1 on 3 AR reporters.

	PSA 6.1-Luc	PB-Luc	ARR3-Luc
SINT1 IC_{50} (µM)	10.74 ± 2.44	11.70 ± 1.82	13.32 ± 0.93

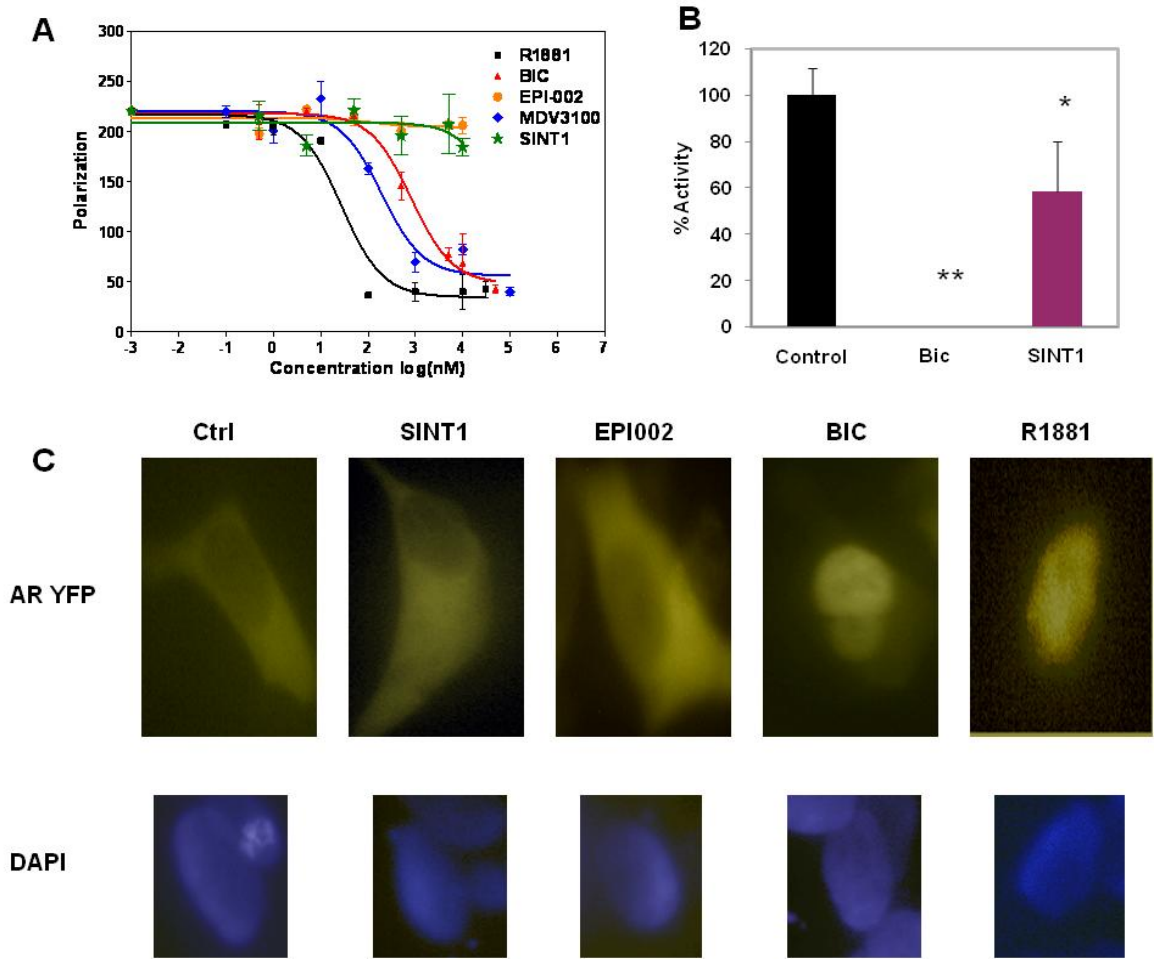

Figure 8. SINT1 has a unique mechanism of action compared to antiandrogens and blocks AR N/C interaction.

A) *In vitro* ligand competition binding curve showing displacement of 1nM fluorescently labeled ligand (Fluormone™ AL Green) from recombinant AR-LBD (25nM) by agonist R1881, bicalutamide (BIC), MDV3100 but not SINT1 or EPI-002. Data representative of 2 experiments.

B) SINT1 blocks N/C interaction induced by androgen. CV1 cells transfected with GAL4-AR DBD and/or VP16-ARTAD and Gal4-luciferase reporter. Cells were pretreated for 1 hr with BIC or SINT1 before addition of R1881 for 24 hr. Y axis indicating % activity, calculated based on luciferase activity (RLU/mg protein/min) of treatment groups normalized to the control. The error bars represent the mean ± SEM. Data average of 3 experiments. Student's t test: $^{**}p < 0.01$ $^{*}p < 0.05$.

C) Nuclear translocation of AR in LNCaP cells transfected with AR-YFP construct in serum free condition for 24 hr prior to treatment of 1nM R1881, 10µM BIC, 10µg/ml EPI-002, or 10 µM SINT1 for 1 hr. DAPI staining was applied to show the location of the nuclei. Data representative of 3 experiments.

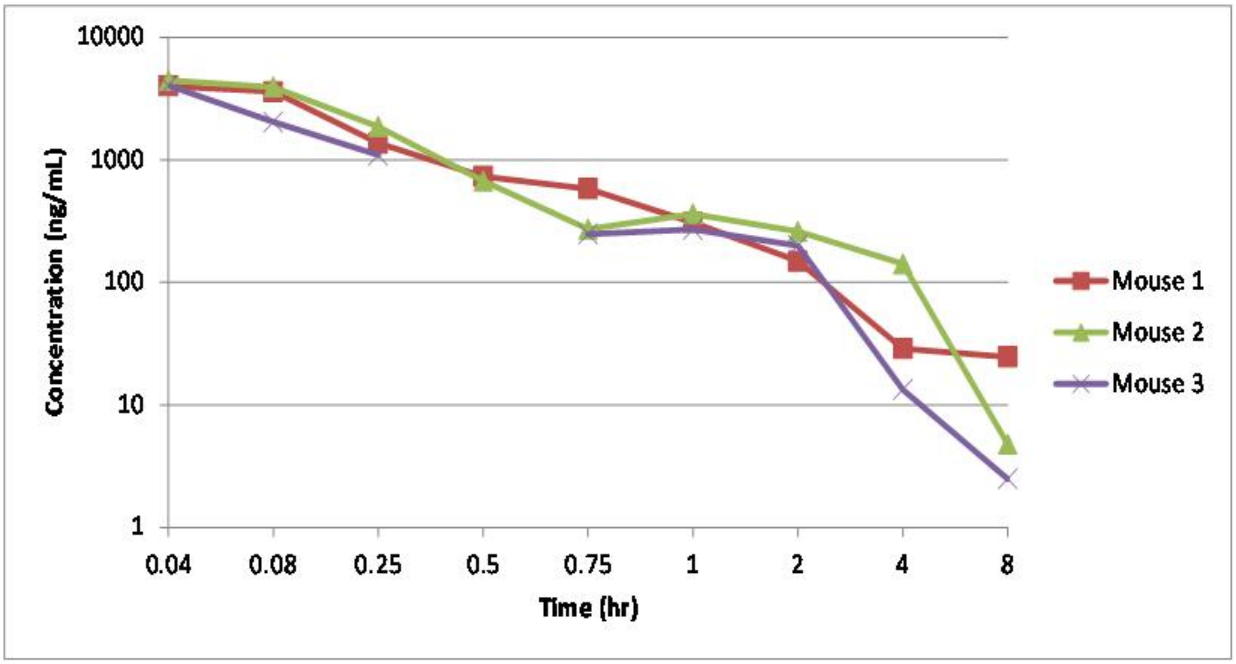

Figure 9. Pharmacokinetics of SINT1.

Plasma concentration of SINT1 after an intravenous dose of 50mg/kg body weight in mice. Three mice were sacrificed per time point for a total of 9 points (2.5min-8hr), and the concentration of SINT1 in the plasma was measured by LC/MS/MS. Mouse 1-3 represent sets of 9 mice (Total n=27). Pharmacokinetics study and data analysis were done at *NAEJA pharmaceuticals Inc.*

Table 2. Pharmacokinetic parameters of SINT1 after an intravenous dose of 50 mg/kg body weight in mice.

PK Parameters	Units	Mice/Set 1	Mice/Set 2	Mice/Set 3	Mean	Std Dev
AUC_{Inf}	hr*ng/mL	1841.5717	2374.353	1509.3933	1908.439	436.340
C_{max}	ng/mL	3990.36	4445.63	4028.39	4154.793	252.589
Cl	mL/hr/kg	21964.9329	17036.2203	26798.8477	21933.334	4881.390
Half Life	hr	1.3777	1.1044	1.0077	1.163	0.192
Vd	mL/kg	43658.5801	27143.634	38960.753	36587.656	8509.380

* Linear trapezoidal method used. C_{max}, peak concentration; Vd, volume of distribution; AUC, area under the plasma concentration-time curve; Cl, clearance volume.

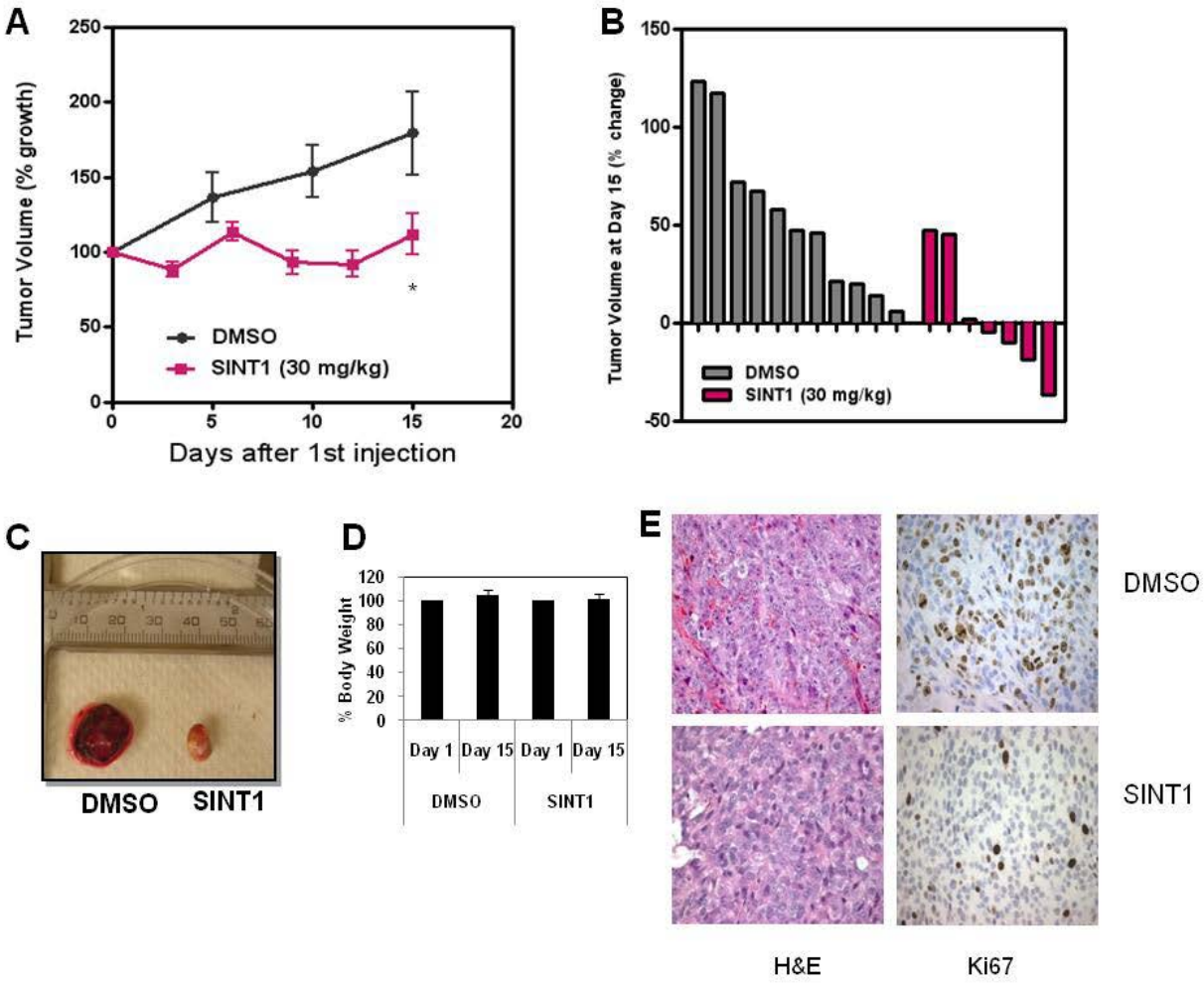

Figure 10. SINT1 causes regression of CRPC xenografts.

A) Tumor growth in SCID mice bearing s.c. tumors receiving intratumoral (i.t.) injections of SINT1 or vehicle control. Mice received i.t. delivery of SINT1 (30 mg/kg body weight) or vehicle that began 7 days after castration. The error bars represent the mean ± SEM. SINT1: Tumor volume mean = 103.3± 11.97 mm^3 , n=7; DMSO: Tumor volume mean = 153.7± 11.87 mm^3 n = 11 (p=0.011). Student's t test: $^*p < 0.05$.

B) Individual animal tumor volume of the same study on day 15.

C) Photographs of the harvested tumors from animals that received SINT1 or vehicle treatment.

D) Body weight of animals on day 1 (1st injection) and day 15. Bars represent the mean ± SEM.

E) Immunohistochemistry (IHC) of representative sections of xenografts harvested at the duration of the experiment and stained for Ki67 and hematoxylin and eosin (H&E). IHC was performed at *Wax-it Histology Services Inc.*

Chapter 3 - Perspectives and Future Directions

3.1 Biological implications of the AR NTD inhibitors

The AR signaling pathway is critical for the development of CRPC. A continuous role of AR as a key player in the hormonal progression of prostate cancer has been suggested recently [28, 33, 290]. Hence the effort for discovery of novel drugs targeting this pathway continues. Within the past 3 years, 5 novel therapeutics have been introduced for CRPC, giving patients more options and survival benefit of a few months [207, 214]. However, resistance to such novel therapies, including abiraterone and MDV3100, is inevitable and unfortunately advanced prostate cancer is not curable.

Recent discovery of constitutively activity AR splice variants lacking the LBD is a proposed mechanism for CRPC [29]. The crucial role of AR NTD in the transcriptional activity of the AR has been confirmed by the discovery of these splice variants [139]. Curiously, studies have also shown that these variants have the capability to function independent of the full-length receptor, and the presence of AR NTD is sufficient for nuclear translocation and transactivation of AR [139, 183, 190, 192]. Hence, recently attention has been driven to AR NTD as a target for development of inhibitors which previously has been understudied [44]. The ID nature of AR NTD has historically made it a difficult target for rational drug design; however recent studies show a promising future for the field of ID proteins due to a better understanding of the mechanism of action of these proteins [291]. AR NTD's signature characteristics include its interaction with multiple partners, its role as a hub for cofactor recruitment, and more recently evidence of its druggability with the discovery of EPI-001 [102, 122]. Based on the studies on

other ID proteins, such as P53 and c-myc [239-241], we hypothesized that AR NTD also possesses ‘hot spots’ that are sensitive regions with binding capacity for small molecule drugs and possibility peptide compounds such as SINT1. These hot spots are believed to be capable of binding drugs independently and simultaneously, further emphasizing the potential of NTD targeting for blocking AR activity. In this thesis, proof of principle studies demonstrated that SINT1 targets the NTD and blocks transcriptional activity of the full-length receptor. However, as novel inhibitors of AR NTD are introduced, a fundamental requirement would be to understand the molecular mechanism of the activity of these inhibitors. As mentioned earlier, the region of the NTD that these compounds bind in order to inhibit the receptor activity is of great interest. Identification of the NTD residues that are functionally critical and are possible targets for drug development, the so called hot spots of IDPs, would pave the way for the development of the next generation of AR NTD inhibitors. Both SINT1 and EPI-001 inhibit the transcriptional activity of the AR in response to ligand, which requires the NTD AF-1 (Tau1) between residues 101 and 370, and transcription in the absence of androgens, which requires Tau5 between residues 360 and 485 [122, 292]. This suggests that binding of these inhibitors to NTD affects the general structure of the receptor rather than specific and regional changes. One possibility is induction of a collapsed folding that would disrupt multiple protein interactions [245, 293]. Metallo and colleagues have shown that small molecule drugs could directly bind the hot spots on c-myc [294]. They also demonstrate that this binding changes the overall disorder state of the ID region to a more stable conformation and replaces the need for the partner protein binding to create the stabilized state; and hence blocks the protein-protein interactions [241, 244].

The effect of peptide inhibitors as opposed to small molecules on IDP is yet to be studied. The smaller size of SINT1 compared to common peptide inhibitors (commonly higher than 10

amino acids) suggests that this inhibitor's interaction with the NTD could mimic similar conformation changes as those induced by small molecule inhibitors. However this effect needs to be studied in more details in future studies. If such explanation holds true, the induced folding of AR NTD might be a step towards analysis of structure of this region by advanced bioinformatics and computational applications that have recently become available for characterization and prediction of IDP [188, 295]. Even though crystallization of the inhibitor bound protein may not be possible, IDPs could be classified into groups based on mechanisms of their conformational changes [242]. An accurate evaluation of the IDP flexibility could also be made based on physics theories [296, 297]. With more structural data available, rational design of next AR NTD inhibitors would be a possibility in the near future [243]. Identification of different classes of NTD inhibitors would also enhance the discovery of multiple binding sites on this IDP. With the evidence that the binding sites for multiple small molecule or possibly peptide inhibitors exist on the NTD, discovery of various classes of inhibitors for NTD could follow, similar to the currently available LBD inhibitors. Such discoveries would greatly increase the options for CRPC management clinically and provide patients with the next generation of drugs for treatment of advanced prostate cancer.

Inhibition of protein-protein interactions is the proposed mechanism of AR NTD inhibitors such as EPI-001 [122]; hence studying alterations in these interactions could provide information on the structural and molecular mechanisms underlying the NTD inhibitors. Although it is critical to investigate the effect of SINT1 and EPI-001 on common co-activators and co-repressors that regulate activity of most nuclear receptors, such as CBP and SRCs, it is also important to consider the unique characteristics of AR NTD, in the context of an IDP. The rationale for the specificity of NTD inhibitors is the low homology between the NTD of the AR

and other nuclear hormone receptors [44, 298]. This highlights the existence and importance of protein interactions that are unique and specific to the NTD which must be studied and approved as valid targets blocked by NTD inhibitors. With a perspective highlighting the unique characteristics of AR NTD and its IDP nature, alterations in protein interactions between AR and membrane associated proteins such as tyrosine kinase receptors (eg. IGFR1) [299], and/or cytoplasmic co-regulatory proteins, in addition to transcriptional cofactors would be assessed. Probable mechanisms for drug activity could include blocking protein-protein interactions involved in regulation of cell volume, membrane transport channels, splicing factors, and/or apoptosis pathways. As AR is known to interact with at least 169 different proteins [97], identification of all protein interactions blocked by EPI-001 or SINT1 could be a challenging task. Hence, as previously discussed, high-throughput approaches by CoIP MS techniques would be one way to investigate this issue. This approach would be an unbiased study to identify all protein-interactions as targets for inhibitor activity [96, 193]. This method would also be helpful for assessing the differences between mechanism of EPI-001 and SINT1 activities. Discovery of common characteristics in the identified proteins could also elucidate the most critical protein-protein interactions among various classes of inhibitors.

Given the promising preclinical results for EPI-001 and SINT1, the next stage of drug development would be to test the efficacy and safety of these drugs in other animal species and ultimately in humans in clinical trials. One important factor would be the selection of the target population niche for the AR NTD inhibitors. To do so, efficacy and safety of these compounds compared to the current approved drugs must be determined. Whether inhibitors of this ID region of AR would work with the same consistency and potency as observed in the pre-clinical studies needs to be determined in the clinical studies. One may hypothesize that patient

populations that are shown to express both full-length AR and the constitutively active splice variants, as well as the ones that present with metastases that solely express AR variants such as AR^{v567}, would be appropriate target niches for NTD inhibitors [192]. Comparing the effect of NTD inhibitors with antiandrogens such as MDV3100 in various patient populations and stages of the disease would also provide the key information for selection of the most efficacious treatment for the patients. However, a challenging task in the initial design of clinical investigation of NTD inhibitors would be to consider the novel treatments that are now available for CRPC patients and the probable resistance mechanisms emerging in the patients post treatments with such agents (e.g. abiraterone and MDV3100) [207]. For a comprehensive assessment of the activity of NTD inhibitors, the above factors must be taken into consideration when designing the clinical trials.

With approval of novel therapies, patients with advanced prostate cancer could be offered more treatment options. However, clinicians are now facing the challenge of optimizing care by selecting the most beneficial treatment. Treatments with each of the inhibitors of androgen/ AR axis and the order in which these therapies are administered may affect overall survival of patients. Of importance is the mechanism of resistance to each of these novel therapies as this could be the determining factor for the next treatment. In the case of abiraterone acetate, tumors post treatments are difficult to treat [207, 300]. Interestingly, recent evidence has shown that abiraterone could not only inhibit the androgen synthesis by targeting the CYP17 enzyme, it also has some anti-androgen activity. With this combination of inhibitory activities, it is believed that tumors capable of survival under conditions of low levels of androgen and AR blockade are selected for which are then difficult to treat with current therapies. Consistent with this explanation, mechanism of resistant to abiraterone includes increased expression of AR splice

variants which are constitutively active and do not rely on androgen synthesis [220]. This could be one explanation for the difficulty of treatment of patients post abiraterone therapy. In such a case, inhibitors of AR NTD, including EPI-001 and SINT1, are theoretically the only known AR antagonists that would have beneficial results for treating those patients with constitutively active AR splice variants.

Historically, AR antagonists targeting the LBD fail due to mechanisms including AR mutants and alterations in levels of expression of co-regulatory proteins. Although mechanisms of resistance to MDV3100 are only just emerging, selection for AR variant expression as a resistance mechanism appears plausible based on induced levels of expression of variants *in vitro* [301]. Similar to the abiraterone resistant situation, treatment with EPI-001 or SINT1 and other inhibitors of AR NTD is expected to show beneficial results for patients in the case of MDV3100 resistance. Combination therapy has also been an interesting suggestion. Combination therapy with MDV3100 and abiraterone acetate has been proposed and might undergo clinical trials in the near future [302]. Combination therapies using an antiandrogen, such as MDV3100 combined with EPI-001 or SINT1, may yield synergistic or additive responses in patients with multiple tumors with varying ratios of full-length AR and splice variants.

Understanding the underlying mechanism of activity of NTD inhibitors is critical in prediction and preparation for the possible resistance mechanisms to these inhibitors. Gain of function point mutations in the AR NTD, similar to the mutations in the LBD following treatment with antiandrogens, are less probable due to the ID nature of region, and such mutations are expected to have less implications in the function of the protein. Interestingly, the unique mechanism of activity of these inhibitors is believed to lead to an entirely new phenotype of resistant tumors not previously observed. Considering the ID nature of this molecular target

and the possibility of interaction with multiple partner proteins, increase in expression level of co-activators/decrease in co-repressors, conformational changes in the NTD to allow for interaction with novel partner proteins, hyper-sensitization of the NTD through increased PTM such as phosphorylation, and posttranscriptional modification to the AR transcript could be possible mechanisms of resistance to these novel inhibitors. Hence, the next steps in studying AR NTD inhibitors would be to design studies to characterize and overcome such mechanisms and ultimately, prepare for targeting the resistant tumors with novel phenotypes.

To put the overall impact of the discovery of novel AR NTD inhibitors in perspective, it is critical to consider that ADT and antiandrogens such as bicalutamide have been used for treating prostate cancer patients for decades. Although beneficial for the patients, the underlying molecular mechanism of most antiandrogens has been targeting the LBD of the AR, as a result of which, some of the current resistance mechanisms in CRPC have developed; hence the need for a novel target in drug development. AR NTD inhibitors are the first group of compounds that are designed for inhibition of a novel molecular target. Using these compounds, solely and possibly in combination with antiandrogens would raise the possibility of complete inhibition of AR signaling, and ultimately remission of disease in CRPC patients. Hence, this is an exciting point in time for AR signaling researchers, as well as prostate cancer patients.

Complete remission of the disease could also be made possible through targeting multiple signaling pathways within the cells. Dysregulation of phosphatidylinositol 3-kinases (PI3K), commonly through loss of phosphatase and tensin homolog (PTEN) in cancer cells, and emergence of other signaling pathways such as Src kinase for metastasis, are also among the well studied pathways of advanced prostate cancer [303, 304]. Inhibitors of targets in such pathways are also under development. Hence, an accurate knowledge of the molecular stage of the

disease, as well as combination of all possible treatments would be an integral part of treatment for prostate cancer patients. Consistent with this observation, an interesting new study has employed a shRNA library screen for identification of new targets in CRPC models [305]. As survival and growth pathways and molecular targets could be redundant in multiple cancers, identification of novel valid targets with existing inhibitors would provide a fast track approach for treatment of patients who cannot benefit from current therapies.

In another approach, microRNAs, important regulators of biological systems, have been used as molecular targets [306]. It is suggested that miR-21 plays an essential role in regulation of apoptosis and metastasis of prostate cancer. This microRNA is known to down regulate PTEN and AKT signaling pathway which is consistent with the possible mechanisms of bypassing AR signaling [307]. Therapies targeting miR-21, or other microRNAs with known functions in CRPC progression would be a potential alternative treatment for CRPC patients with AR bypass mechanisms.

Additionally, novel antiandrogens are under investigation for treatment of other cancer types such as breast and ovarian tumors. AR has been implicated in breast carcinogenesis and is expressed in about 70% of breast cancers [308]. One subtype of breast cancer known as ‘triple negative’ tumors lacking progesterone, estrogen and HER2 receptors are of particular interest. Due to lack of receptors, no targeted endocrine therapy is currently available for these patients, which are approximately 20 to 30% of all breast cancers [309]. These patients commonly represent a more aggressive clinical course of the disease. Recent evidence has revealed an important role for AR signaling in these tumors, hence AR inhibitors could be promising therapies for this patient population. Effect of MDV3100 for this patient population is currently

under investigation. AR NTD inhibitors, EPI-001 and SINT1, could be used for AR targeting in other cancer types as well.

Despite the advances in drug development and discovery of novel therapeutics in the last few years, the complete mechanisms of progression of prostate cancer to CRPC are yet to be elucidated. A few outstanding questions regarding such mechanisms would be:

- How would low androgen levels signal for alternative splicing and/or DNA rearrangements, that create AR splice variants? How are these processes regulated and linked? Could microRNAs have a role in translation of this shift from low androgen levels to AR variant expressions?
- The two mechanisms of CRPC breakthrough, *de novo* androgen synthesis and AR splice variant expression, seem to be emphasizing two opposite mechanisms for cell survival; the former requiring AR full-length activity with the presence of functional LBD, and the latter coding for AR species lacking LBD with constitutive activity. Which mechanism would be selected for first (if an order exists)? And are these mechanisms reversible or interchangeable? Would it be energetically favorable for the cancer cells to employ both?

3.2 Drug development perspectives

Approximately 140 peptide drugs are being developed as clinical drug candidates. In 2010, the sales of 60 approved peptide drugs was about $13 billion [310, 311]. These drugs are used as therapeutic agents for conditions including oncology, metabolic disorders, and cardiovascular diseases, with a large number of molecular targets being extracellular G-protein-coupled receptors. Examples of peptide drugs are glatiramer acetate (GA, Copaxone©, Teva

Pharmaceuticals, Petah Tikva, Israel) [312], a drug for multiple sclerosis therapy and natural peptides such as insulin, vancomycin, oxytocin, and cyclosporine. Interestingly, about a half of the approved peptide drugs are based on natural products. The important advantages offered by peptide drugs are high specificity, potency, and low toxicity [255]. However, practical issues such as reduced stability and shorter half-life must also be considered. Hence, a successful drug development process needs to be guided by an accurate estimate of the pharmacokinetic data, such as bioavailability and half-life, and the factors that affect these parameters [251]. Achieving these criteria has been made possible through advances in medicinal chemistry methodologies. Such methodologies allow for obtaining desired structural and pharmacological properties like charge or lipophilicity. Through modifying these factors, optimal drug absorption and disposition could be achieved. As an example, amino acids could be replaced with functionalized or non-natural substituted blocks to improve a peptide's stability, activity, and half-life. Interestingly, to make larger peptides more resistant to proteolysis, synthesizing the peptide sequence in reverse order using d-amino acids has been used [254]. In recent years, clinicians and patients have also been more open to injected therapies, which would be the ideal delivery method overcoming the practical hurdles of peptide drugs.

Advances in delivery technology, such as lipid nano particles (LNP), that would allow for encapsulation of the drug and enhanced stability and target specificity could also have a major impact on peptide drug development [313, 314].

As discussed in this thesis, SINT1 is a natural compound with potential advantages and disadvantages for drug development. The small size and faster clearance of SINT1 could translate to a less significant immune response to this compound. A clinical candidate with low immunogenicity would have an advantage of being tolerated as a single agent without the need

for GR agonists such as prednisone. However, faster metabolism by the liver or clearing from the circulation through the kidneys would also affect the efficiency of the drug due to shorter exposure of the tumor to the drug. Hence the expected efficacy may only be achieved at higher doses or through more frequent administration. As discussed above, chemical modification to the backbone structure of SINT1 could increase the stability of the compound and enhance its solubility to facilitate higher dosing options. These studies are now under intense investigation.

Another critical factor associated with natural compounds is the limited sources of the drugs. As sponges that SINT1 was isolated from were collected from ocean waters a few years ago, there are limited samples available for biological studies. This emphasizes the need for a synthetic compound which can be produced in larger scale and is as potent as the natural compound. Synthesis of SINT1, specifically at large scale, has been difficult as expected for peptide molecules; however studies optimizing multiple steps for synthesizing this peptide and those with modified structures are ongoing [315]. Our team and collaborators are currently investigating the synthetic SINT1 analogues, and structure activity relationship (SAR) studies are aimed to select the most potent drug for future studies.

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